EPIDEMIOLOGY OF INTESTINAL PARASITES IN RELATION TO HIV INFECTION IN WESTERN KENYA WITH SPECIAL REFERENCE TO *CRYPTOSPORIDIUM*

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by

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ABSTRACT

Examination of 758 faecal samples from mothers and children attending a Mother To Child Transmission (MTCT)-HIV study on gastrointestinal parasites was undertaken between June 1999 and January 2000 in western Kenya. Seven of the most common non-opportunistic protozoa and three of the common helminths were recorded. *Entamoeba coli* was the most frequent being identified in 52% of the samples. Others were *Entamoeba histolytica/E. dispar* (19.3%), *Endolimax nana* (9.4%), *Giardia lamblia* (9.4%), *Chilomastix mesnili* (16.9%), *Blastocystis hominis* (9.6%), *Iodamoeba buetschlii* (10.4%), *Ascaris lumbricoides* (23.2%), Hookworms (18.7%), and *Trichuris trichiura* (3.6%). Parasite prevalence was higher in mothers than children, and similar in all the villages surveyed over the three examinations. Polyparasitism was common with a mean number of 2.1 different species per person among mothers and 1.2 in children. HIV-infected mothers were no more likely to be infected with individual parasites than HIV-uninfected ones (OR1.5, 95 %CI 0.832-2.252). HIV-infected children were at an increased risk of hookworm infestation (OR 2.333, 95% CI 1046-5.203; p=0.032)

A measure of transmission mechanisms for the common parasites in the community was done by the application of Bekessy's model that estimates daily incidence and clearance rates. The longest estimate for duration of infection was for *E. coli* with 225 days in mothers, while the shortest was 38 days for *B. hominis*. The model revealed specific parasites had different duration in either mothers or children that corresponded to their varying prevalence in the two groups. Adjusting levels of parasite detectability assessed the reliability of the model. The adjustments revealed transmission estimates for duration of infections were robust even from marginal longitudinal prevalence data.

A7% *Cryptosporidium* prevalence was recorded by acid-fast staining of faecal samples from the MTCT cohort. Genotype identification targeting the hyper-variable region of the 18S rRNA gene of *Cryptosporidium* was undertaken. The results were compared to those of samples recovered from persons living in Malawi, Brazil, Vietnam, UK and Thailand with or without HIV infection. *Cryptosporidium* genotypes that included *C. parvum* 'human' genotype, *C. parvum* 'bovine' genotype, *C. meleagridis*, *C. felis*, *C. muris* and *C. canis* were identified. Majority of isolates from all areas were identified as *C. parvum* 'human' or 'bovine' genotypes. Nucleotide diversity was most extensive in the *C. parvum* 'human' genotype that had significantly higher singleton mutation rates of 26.13 compared to all the other genotypes with singleton mutation rates varying from 5.3 to 6.86 (p=0.01). *Cryptosporidium muris* infection was confirmed for the first time in a human patient from Kenya using molecular techniques. Zoonotic species, specifically *C. parvum* 'bovine' genotype, *C. meleagridis, C. felis* and *C. canis* were also detected in isolates from HIV-positive patients from Thailand. In total, six *Cryptosporidium* genotypes were identified, the highest diversity occurring in HIV-infected people regardless of their geographical location.

To the children of western Kenya who took part in this study

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
Al	*Ascaris lumbricoides*
ANOVA	Analysis of variance one way
AZT	azido-deoxythymidine, zidovudine
Blast	blast local alignment search tool
BDH	Bund Deutscher Heilpraktiker ®
Bh	*Blastocystis hominis*
Bz	Brazil
CD4	Cluster of differentiation 4 T-lymphocytes
CDC	Centers for Disease Control and Prevention
cdc2	cyclin-dependent protein kinase gene
Ch-	Children HIV-uninfected
Ch+	Children HIV-infected
CI	Confidence interval
Cm	*Chilomastix mesnili*
CMR	Centre for Microbiology Research
COWP	*Cryptosporidium* oocysts wall protein locus
DETR	Department of Environment Transport and Regions (UK)
DHFR-TS	Dihydrofololate reductase thymidylate synthase
DNA	Dioxyribonucleic acid
dNTP	2'-deoxynucleotide 5' triphosphate
DR Congo	Democratic Republic of Congo
DWI	Drinking Water Inspectorate
Ec	*Entamoeba coli*
EDTA	Ethylenediamine tetraacetate
EF-1α	Elongation factor-1α gene
Eh	*Entamoeba histolytica*
En	*Endolimax nana*
EPA	Environmental Protection Agency (USA)
Gl	*Giardia lamblia*
HAART	Highly active antiretroviral therapy
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HSP70	Heat shock protein (70kilodalton) gene
Hw	Hookworms
Ib	*Iodamoeba buetschlii*
IgE	Immunoglobulin E
IPTG	isopropyl-β-D-galactopyranoside
ITS	Internal transcriber spacer
JICA	Japanese International Cooperation Agency
KAP	Knowledge attitudes and practices
Ke	Kenya
KEMRI	Kenya Medical Research Institute

LB	Laurier broth
LBA	Long branch artefact
LSU	Long subunit gene
M-	Mothers HIV-uninfected
M+	Mothers HIV-infected
MgCl	Magnesium Chloride
MIF	Merthiolate-iodine-formaldehyde
MS	Microsoft
MTCT	Mother to child transmission
Mw	Malawi
NaCl	Sodium Chloride
NY	New York
OR	Odds ratio
PCR	Polymerase chain reaction
PE	Perkin Elmer
pGEMT	Vector system (Promega)
polyT	polythreonine locus
PVA	Polyvinyl alcohol
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNR	Ribonucleotide reductase
rRNA	ribosomal ribonucleic acid
SAF	Sodium acetate formalin
SD	Standard Deviation
SPSS	Statistical package for Social Scientists
*Ssp*1	*Sphaerotilus* derived endonuclease
SSU	Small subunit
Taq	*Thermus aquaticus* derived polymerase
TBE	Tris-borate EDTA
TG2	Transformation system bacteria
Th	Thailand
Th2	Thymus derived helper 2 cells
TNFα	Tumour necrosis factor-α
TRAP-C1	Thrombospondin-related adhesive protein 1 of *Cryptosporidium*
TRAP-C2	Thrombospondin-related adhesive protein 2 of *Cryptosporidium*
tsv	total sample volume
Tt	*Trichuris trichiura*
UK	United Kingdom
USA	United States of America
*Vsp*1	*Arthobacter* derived endonuclease
Vt	Vietnam
WHO	World Health Organisation
w/v	weight by volume
ZN	Zeihl Neelsen

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To the children of western Kenya who took part in this study

Summary

While the occurrence and control measures for intestinal parasites are well documented, there is less information on the patterns of infections in communities at risk especially in the wake of HIV/AIDS infection. The aim of our study was to investigate the intestinal parasite profiles, prevalence, incidence and transmission mechanisms in relation to the HIV status of the participants.

The study area is geographically a tropical hot lake basin with a varying hinterland that may be hot and dry, or hot and humid. Our study cohort was a group of mothers and their children enrolled in a larger follow-up study of Mother to Child Transmission (MTCT) of HIV. Ethical clearance was obtained and individual consent was sought for every participating adult, for themselves and on behalf of their children. Where possible the participants were examined at the beginning of the survey and thereafter after 3 and 6 months.

The survey identified the presence of at least 10 of the common protozoa and helminths. These were *Entamoeba coli*, *Entamoeba histolytica/Entamoeba dispar*, *Endolimax nana*, *Giardia lamblia, Chilomastix mesnili*, *Blastocystis hominis*, *Iodamoeba buetschlii*, for the protozoa while the helminths were *Ascaris lumbricoides*, hookworms, and *Trichuris trichiura*. Their prevalence remained stable over the period of seven months. *E. coli* was the most commonly identified parasite with an average of 63% and 31% prevalence in mothers and children respectively. Infection with *A. lumbricoides* was high as expected in this area, with an average of 24% in mothers and 19.4% in children. Follow-up survey revealed a significant

increase in three and six month's period prevalence in both mothers and children indicating contamination and high transmission rate for parasites with a faecal/oral spread. At least one parasite was identified in more than 77% (584/758) of all the samples examined throughout the study while over 50% (379/758) had more than two parasites.

The above epidemiological factors were investigated in relation to HIV status of the mothers and children enrolled in the study. Overall, the HIV status did not appear to affect the pattern of infection for most individual parasites except in the case of hookworm infections. HIV-infected children were significantly at a higher risk to hookworm infection than uninfected children (OR 2.333, 95% CI 1046-5.203; p=0.032). No other factors seemed to increase the risk to hookworm infections in either mothers or children.

Transmission mechanisms in the community were investigated by the analysis of crude incidence and clearance rates for the most common parasites in mothers. A three months incidence of some of the common parasites ranged from a high of 48.8% in *E. coli* to a low of 6.4 % for *G. lamblia* in mothers. Results showed children had a *G. lamblia* three months incidence of 21.4%, while incidence was lowest for *I. buetschlii* among the protozoa at 4.3%. Three months clearance rates in mothers were highest in infections with *B. hominis* (83.3%), *E. histolytica* (71.4%) and *G. lamblia* (75%) among the protozoa while *T. trichiura* recorded a 100% three months clearance rate. Three months clearance rate was higher in children at 83.3% in *E. histolytica*, 84.3% for *E. nana* and 66.7% for *G. lamblia* while *E. coli* had lower

clearance rate of 41.7% than incidence (48.7%) for the same period. However interpretation of the crude incidence and clearance rate to the occurrence of parasites in the community seemed to underestimate infection rates and consequently the overall prevalence.

In this regard, further estimates were made using a model described by Bekessy and others (1976) that estimates daily transition rates relating to incidence and clearance rates. The model describes the simultaneous estimation of daily incidence and recovery rates from a longitudinal study of positive and negative infections in a defined cohort, allowing for several transitions between consecutive surveys. The estimates are then used to compute duration of infections or durations between infections. Using this model, *E. coli*, showed the longest duration of infection of about 225 days in mothers. The parasite showed a daily incidence of 0.00953 infections per person, and a daily clearance rate of 0.00445, which is almost half the rate of daily new infections. The rates corresponded to the observed prevalence of *E. coli* that showed gradual increase in period prevalence where incidence was higher than clearance rates.

In contrast *G. lamblia* with a lower prevalence of 5.7 % had a daily incidence of 0.00147 and a daily clearance rate of 0.0172, the latter almost 11 times higher than daily incidence. The estimated duration of infection in mothers was 58 days. This was in contrast to the observed transition rates for *E. coli*. While *E. coli* and *G. lamblia* have similar transmission mechanisms, their differences in transition rates and duration of infections resulted in the contrasting prevalence in the community.

Each parasite's duration of infection coupled with the different clearance rates and re-infection determines the clusters of infected people where exposure is assumed to be similar.

Adjusting the prevalence results for missed infections by assuming higher parasite detectability assessed the reliability of the model in the estimates of transition rates. This adjustment demonstrated the robustness of duration of infection estimates in low sensitivity of parasite identification. There was no significant change in the estimated duration of infection for *E. coli* assuming a 10, 30 and 50% increase in parasite detectability. The adjustments resulted to duration of infections ranging from 219, 212 and 234 days compared to our original estimates of 225 days.

In the second phase of the study, *Cryptosporidium* parasites were identified by acid-fast staining from a selection of stool samples of the participants in the MTCT study. *Cryptosporidium* is a significant cause of morbidity and mortality especially in HIV/AIDS patients. Samples were preserved either in 2.5% potassium dichromate, frozen fresh at -80^0C or cooled at 4 ^{0}C. Combination of different methods revealed the highest success rate for *Cryptosporidium* DNA extraction and amplification, was from samples without preservatives frozen at -80^0C. A modification of the extraction method where the lysis buffer from the stool DNA extraction kit was incorporated in the freeze/thaw process to rupture the oocysts increased the recovery of *Cryptosporidium* DNA from the stool samples. Genotypic analysis of the parasites' DNA was done by restriction fragment length polymorphism on the highly

polymorphic fragment of the 18S rRNA gene, and thereafter confirmed by sequencing.

A total of six *Cryptosporidium* genotypes were identified in the isolates analysed. Over 80% of all isolates were identified as either *C. parvum* 'human' (genotype 1) or *C. parvum* 'bovine' genotype (genotype 2). Significantly, *C. muris*, hitherto, a primarily murine parasite but also infecting other mammals was identified in an HIV-infected patient with clinical symptoms from Kenya. *C. meleagridis* was identified in an HIV patient from Kenya and in 20% of isolates from Thailand. Almost 50% of isolates from Thailand were of zoonotic type that included *C. parvum* 'bovine' genotype, *C. felis* and *C. canis*. This is the first report to the best of our knowledge, identifying *C. parvum* 'bovine' genotype and *C. canis* and the second reporting the occurrence of *C. meleagridis* and *C. felis* from human isolates recovered from HIV-infected patients living in Thailand. Apart from *C. parvum* 'bovine' genotype, all zoonotic species of *Cryptosporidium* were identified in HIV-infected individuals. There was no intra-genotypic difference in the species identified by age, geographical origin or by HIV status.

Genetic relatedness of the isolates from the different geographical sources was assessed by phylogeny at the 18S rRNA gene locus. The resulting phylogram inferred a tree with similar topology to that from isolates recovered from natural (animal) hosts. There was clear distinction of the different species and genotypes with *C. muris* showing the furthest evolutionary distance of over 6% from all the other species. The highest intra-genotypic variation was observed in the *C. parvum*

genotype. This was shown to be due to high singleton mutations that was most extensive in *C. parvum* 'human' genotype (Eta = 27) compared to the singleton mutation sites in all other species (Eta = 7-8) New strains of *C. meleagridis* and *C. felis* that had different restriction profiles from any of the published strains were identified. Again nucleotide diversity did not vary by HIV status or geographical origin.

1.0 Chapter 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Epidemiology of intestinal parasites

1.1.1 Parasitism

Parasitism as an ecological relationship is the most prevalent life style among organisms (Beaver *et al.,* 1984; Bush *et al.,* 2001). It refers to a reciprocal association in which one species; the host, is injured to some degree by the presence and activities of another, the parasite. The parasite derives shelter and nutrition from the host while the host shows either no visible effect or various degrees of functional and organic disturbance (Belding, 1965). Parasites may be facultative when they lead both a free and parasitic existence or obligate when they completely depend on the host. Occurrence of parasites in general, depends on their endemicity; the presence and habits of suitable hosts, distribution in the environment and colonisation of new hosts. More than 400 species of parasites are known to infect humans, inhabiting all organs of the body, most of them rare (Ashford and Crewe, 1998). Intestinal parasites are either protozoa or helminths, and while some protozoa are commensal, all parasitic stages of helminths are considered pathogenic (WHO, 1981).

However, there is evidence that the presence of certain number of parasites may offer some protective characteristics in the overall health of a host community (Medley *et al.,* 1993). The long association between humans and worms is thought to have evolved to some degree, into a symbiotic relationship (Bundy *et al.,* 1997). Some opinion suggest helminthic infections help in reducing the occurrence of some allergic and inflammatory conditions through the high levels of non-specific IgE and down regulation of T-helper cell type 2 (Th2) (Nelson, 1992; Garside, 2000; Bundy *et al.,* 2000). It is also known that continuous low level exposure to parasites from an early age leads to the development and maintenance of age-related immunity that reduces overall parasitic morbidity in a community (Bundy, 1997; Anderson, 1998; Crompton, 1999). Moreover, some stages of most intestinal parasites are free living. This has led to suggestions that the term 'parasite' refers to individual organisms that, at a given time and to a certain degree depend on, and injure a host, rather than to a whole species (Ashford and Crewe, 1998; Bush *et al.,* 2001).

1.1.2 General Significance

Intestinal parasites are a leading cause of chronic infections in humans worldwide, with estimates showing that at least one quarter of the world population is infected (Bundy, 1997; WHO 1999). Since they mainly occur as mixed intestinal infections, they exacerbate co-existing diseases or conditions such as malnutrition thus, contribute to poor health and impaire cognitive functions (Stephenson, 1980; WHO, 1981; Stephenson, 1994; Schmunis and Antunano, 1998). Intestinal parasites present a major public health problem in the developing world where the scale of infections

far exceeds any economic, technical and social resources for effective control (Buck *et al.,* 1978I; World Bank, 1993).

In the developing countries over 80% of all deaths recorded are due to infectious and parasitic diseases accounting for more than 13 million deaths annually (WHO, 1999). In most cases, a large proportion of the population is susceptible, although mortality and morbidity are more pronounced among the poor. Poverty as measured in lack of sanitation, illiteracy and overcrowding thus becomes a vicious cause and consequence of parasitic diseases in most communities (World Bank, 1993; Albonico *et al.,* 1999). Due to the ubiquitous nature of intestinal parasite infections sometimes with no apparent disease, it is difficult to justify allocation of meagre resources for sustained parasite control programs necessary for their eradication. The situation is worsened by the fact that most countries have little evidence demonstrating direct link between the highly prevalent parasitic infections, and, feasible negative effects on daily subsistence activities of the population (Buck *et al.,* 1978I; Bundy, 1997).

The burden of disease contributed by infectious diseases in Africa is disproportionately high mainly due to malaria, acute respiratory infections, diarrhoeal diseases and intestinal parasites (Rosenfield, *et al.,* 1984). Malaria and schistosomiasis continue to be the leading causes of parasitic morbidity with intestinal parasites a constant underlying factor, the two groups, in association, worsen parasitic morbidity and mortality (World Bank, 1993; Schmunis and Antunano, 1998).

1.1.3 Distribution

Protozoa and helminthic intestinal infections are estimated to affect 3.5 billion people worldwide, the majority being children (Bundy *et al.,* 1997). Some estimates suggest that approximately 1.4 billion people are infected by *Ascaris* worldwide, 1.4 billion with hookworm, 1.1 billion with *Trichuris* and 200 million with *Strongyloides* (Chan *et al*., 1994; Crompton *et al.,* 1999; WHO, 1999). Amoebiasis in the second most common cause of death due to a protozoan parasite worldwide (WHO, 1985; WHO, 1987). Of the 48 million people estimated to suffer from severe amoebiasis 100,000 die annually (WHO; 1987; Espinosa-Cantellano and Martinéz-Palomo 2001). Despite the overall improvements in sanitation, the total number of people infected with parasites word-wide is thought to be increasing (Beaver, 1975; Chunge *et al*., 1985; Rahman *et al.,* 1994; Bundy, 1997; Curtis, *et al.,* 2000; Scolari *et al.,* 2000).

Intestinal parasites are ubiquitous, although much disparity in prevalence exists between and within specific communities (Walsh and Warren, 1979; Petney and Andrews, 1998; Schmunis and Antunano, 1998). Unlike other parasitic diseases such as malaria and schistosomiasis that present more specific morbidity, effects of intestinal parasite infestation in specific communities is less clear and more insidious (WHO, 1987). The multiplicity of these parasites in their occurrence render direct individual monitoring expensive and proxy estimates from hospital results may be unreliable (Pawlowski, 1987; Crompton and Savioli 1993; Guyatt *et al*., 1991; Fraser and Craig, 1997).

While intensity of infection reflects morbidity, current direct methods of assessing intensity are expensive and labour intensive (Bundy *et al.*, 1992; Fincham *et al.*, 1998). This has led to new interpretations of the conventional methods used in assessing morbidity with some studies demonstrating that prevalence of infection might be used to predict intensity of infection, hence the prevalence of morbidity (Guyatt *et al.,* 1991; Booth and Bundy, 1995; Guyatt *et al.,* 1999; Brooker *et al.,* 2000; Howard *et al.,* 2001). Other studies have used prevalence studies to estimate incidence, parasite clearance rates, parasite associations and duration of infections (Chunge *et al.,* 1991a; Ashford *et al.,* 1992; Petney and Andrews, 1998; Ziam and Pandey, 2000; Howard *et al.,* 2001). This is significant for intestinal parasites as it would be possible through prevalence studies to predict the distribution of multiple infections and assess the risk of morbidity to a community even when only marginal data is available (Nagelkerke *et al.,* 1990; Guyatt *et al.,* 1991; Booth and Bundy, 1995).

1.1.4 Parasitic Infections in Association to HIV

The emergence of HIV/AIDS has compounded the already existing burden of infectious diseases especially in Africa (Orihel, *et al.,* 1995). Overall, chronic parasitic infections result in potent highly polarized immune responses. This is attributed to in the widespread activation of Th2 cells, blood mononuclear cell proliferation and decreased *in vitro* secretion of B chemokines (Bentwich *et al.,* 1999; Borkow *et al.,* 2000; Borkow *et al.,* 2001). In case of schistosome infection, enhanced Th2 response leads to down-regulation of Th1 derived cytokines and impairs cytotoxic T-lymphocyte responses (Sher *et al.,* 1992; Actor *et al.,* 1993).

These dysregulations apparently makes the host more susceptible to infections by HIV with increased expression of HIV co-receptors (Bentwich *et al.,* 1995). However, these observations while compelling, are inconclusive due to the confounding conditions that would affect immune systems of people living in regions endemic with most infectious diseases (Bundy *et al.,* 2000). Numerous studies have outlined the outcome of HIV co-infections with endemic parasites such as malaria, schistosomiasis, leishmaniasis, trypanosomiasis and onchocerciasis apart from the common intestinal parasites (Table 1.1). While these infectious diseases may be leading causes of morbidity, their distribution and severity is more regional compared to the ubiquitous distribution of intestinal parasites (Bundy *et al.,* 2000).

Fewer studies have investigated the role of intestinal parasites in people with asymptomatic HIV-infection (Esfandiari *et al.,* 1995; Lindo *et al.,* 1998; Fontanet *et al.,* 2000a; Wiwanitkit, 2001). In general, intestinal parasites both protozoa such as the amoebae, *Giardia lamblia* and helminths such as *Ascaris lumbricoides, Trichuris trichiura* and hookworms (except *Strongyloides*) are considered non-opportunistic (Robinson, 1995). However, these infections have an overall outcome effect as co-infections on the health of the hosts. Some studies suggest HIV seropositivity enhances the severity of non-opportunistic infections including amoebic infections through mechanisms that are not yet clear (Aztori *et al.,* 1993; Fatkenheuer *et al.,* 1997; Hahn and Erby, 1999). Others have shown significantly higher percentage among HIV seropositive patients in some parasite prevalence like *Entamoeba histolytica* (26.5%), *Iodamoeba butschlii* (16.9%), *Dientamoeba fragilis* (25.3%), *Blastocystis hominis* (51.8%) but no differences in infection rates with *G. lamblia,*

Entamoeba coli, Chilomastix mesnili and helminths (Mendez *et al.,* 1994). Still, others report lower intestinal parasite prevalence in HIV-infected people (Lindo *et al.,* 1998; Anand *et al.,* 1998). Due to the multiplicity of infections with parasitic diseases, it is difficult to predict actual effects on the overall health of infected persons. The chronicity of HIV infection, and its impact on the host's immunity warrants long term prospective studies to elucidate the specific interactions and outcomes of co-infections in both children and adults.

Table 1.1 Parasitic infections in association with HIV

Country of	Parasites	Effects of Hosts	References
Uganda	*P. falciparum*	Increased frequency, clinical malaria & parasitemia	Whitworth *et al.*, 2000
Malawi, Kenya	*P. falciparum*	Increased risk in primigravida, maternal anaemia	Verhoeff *et al.*, 1999, van Eijk, 2001
Kenya	*S. mansoni*	Reduced egg excretion	Karanja *et al.*, 1997
Ethiopia	*S. mansoni*	"	Fontanet et al, 2000a
Ethiopia	*L. donovani*	Enhanced progression HIV and leishmaniasis	Berhe *et al.*, 1995
Brazil, East Africa	*Trypanosoma*	No effects-African Trypanosomiasis, Enhanced severity in Chagas disease	Dedet and Pratlong, 2000
Uganda	*O. volvulus*	No effects on response to treatment, impaired Antibody response to *Onchocerca* antigen	Fischer *et al.*, 1995; Tawill *et al.*, 1996
Thailand	Intestinal Nematodes	No increase in frequency	Wiwanitkit, 2001
Hondurus	*G. lamblia*, *A. lumbricoides* *T. trichiura*	Less frequency in HIV infected	Lindo *et al.*, 1998
India	Intestinal Nematodes	Less frequent in HIV infected	Anand *et al.*, 1998
Guinea-Bissau	Coccidia	Increased frequency	
	Nematodes & *B. hominis*	No effect by HIV status	Lebbad *et al.*, 2001
Tanzania	Intestinal Nematodes & Coccidia	Higher frequency in HIV infected	Aztori *et al.*, 1993 Tarimo *et al.*, 1996
Ethiopia	Amoebae	Increased frequency, no effects on other intestinal parasite frequency or severity	Fontanet *et al.*, 2000b
	B. hominis	Increased severity	Llibre *et al.*, 1989
Tanzania	*B. hominis*	Increased frequency HIV infected children	Cegielski *et al.*, 1993
South Africa	*T. vaginalis*	Increased severity of pelvic inflammatory disease	Moodley *et al.*, 2002
Zambia	*C. parvum*	Chronic diarrhea, malabsorption	Chintu *et al.*, 1995
Zambia	Coccidia	Enteropathy	Kelly *et al.*, 1997

In contrast, infections with the spore forming enteric protozoa are significantly more pathogenic in immunosuppressed persons and are therefore regarded as opportunistic (Feinberg, 1990). Most of these infections would otherwise be mild or self-limiting in immunocompetent persons. Numerous studies have demonstrated the severity of diseases such as cryptosporidiosis, isosporiasis, toxoplasmosis and microsporidiosis in immunosuppressed patients (Therizol-Ferly *et al.,* 1989; Ortega, 1993; Wittner *et al.,* 1993; Weber *et al.,* 1994; Goodgame, 1996). The resulting enteropathy may be compounded by disseminated disease, depending on the level of immunosuppression (Khumalo-Ngwenya *et al.,* 1994; van Gool *et al.,* 1995; Harries and Gossius, 1996; Morgan *et al.,* 1996; Cahill and Shevchuk, 1996; Heyworth, 1996; Guerin *et al.,* 1997; Lindo *et al.,* 1998; Hart *et al.,* 2000). The mechanism of the enhanced severity in case of immunosuppression is still unclear.

1.2 Biology of intestinal parasites

1.2.1 Protozoa

Protozoa are single cell-like microorganisms recognised separately (from plant, animals and fungi) as subkingdoms. However, opinions differ as to whether they constitute a subkingdom or the diverse organisms belong to different kingdoms as has been suggested lately (Cox 1998; Cox, 2002). Current opinions place these organisms in two separate Kingdoms, Archezoa and Protozoa (Table1.2) but at present, there is no universally accepted scheme of classification (Cox, 1998). Recent changes in the classificification have been made to reflect the findings of extensive molecular and morphological evolutionary research. Microsporidia previously classified under protozoa, are now considered Fungi (Cavalier-Smith, 1998; Hirt *et*

al., 1999; Weiss, 2000). Phylum Amoebozoa contains the members of former phylum Rhizopoda while there are four phyla including Metamonada that constitute the former Mastigophora. Cox and others (2002) have proposed that the term Apicomplexa does not constitute a valid phylum. The term Apicomplexa was adopted to include the genus *Perkinsus* into the phylum Sporozoa (Cavalier-Smith, 1999a; Cavalier-Smith, 1999b). This genus has now been shown not to belong to the phylum as it is more closely related to the dinoflagellates, making the term Apicomplexa invalid, thus, reinstating Sporozoa to a phylum status. The changes are heralding a return to the traditional taxa created a century ago (Cox, 2002). Though much remains unresolved, research with molecular markers is confirming some of the traditional findings and clarifying some controversial issues (Cox, 1998).

Table 1.2 Classification of intestinal parasites in humans

Empire : **Eukaryota**

Kingdom	Phylum	Class	Order	Genus
Archezoa	Metamonada	Trepomonadea	Diplomonadida	*Giardia*
			Enteromonadida	*Enteromonas*
		Retortamonadea	Retortamonadida	*Chilomastix*
	Microspora*	Microsporea	Microsporidia	*Enterocytozoon, Encephalitozoon*
Protozoa	Amoebozoa (Rhizopoda)	Entamoebidea	Euamoebida	*Entamoeba, Endolimax, Iodamoeba,*
		Amoebaea	Acanthopodida	*Acanthamoeba*
	Ciliophora	Litostomatea	Vestibulifera	*Balantidium*
	Parabasalia	Trichomonadea	Trichomonadida	*Dientamoeba, Trichomonas*
	Sporozoa	Coccidea	Eimeriida	*Cryptosporidium, Cyclospora, Isospora, Sarcocystis, Toxoplasma*
Chromista	Bigyra	Blastocystea		*Blastocystis*
			Family	
Animalia	Nemathelminthes	Adenophorea	Trichuridae	*Trichuris*
		Phasmidea	Ancylostomatidae	*Ancylostoma Necator*
			Ascarididae	*Ascaris Toxocara*
			Oxyuroidae	*Enterobius*
			Strongyloididae	*Strongyloides*
	Platyhelminthes	Cestoidea	Taeniidae	*Taenia Echinococcus*
			Hymenolepididae	*Hymenolepis*

*Microsporidia have since been re-classified with Fungi (Hirt *et al.*, 1999, Cox , 2002) Adopted from Cox, 1998

Protozoa are adapted to most environmental conditions as free-living, commensals or parasites of both plants and animals (Belding, 1965). Unlike parasitic forms that require specific environmental conditions, most free-living forms are able to adjust to diverse environments from the hot springs to freezing conditions at all altitudes (Beaver *et al.,* 1994). Critical to their existence and prevalence of protozoa in different habitats is the availability of nutrients. Most are considered saprophytic and depend on available organic substances including bacteria or disintegrating host substances, while some like *E. histolytica,* may digest the hosts' tissue cells (Belding, 1965). To achieve this, many parasitic protozoa have developed complex enzyme systems that enable their survival as facultative, or obligate anaerobes, while the free-living organisms have retained aerobic pathways (Beaver *et al.*, 1984).

1.2.1.1 Amoebae

Amoebae are unicellular non-flagellated organisms using pseudopodia for both feeding and locomotion. The generic name *Entamoeba* was first proposed in 1895 (Casagrandi and Barbagallo) for the two main species infecting humans namely (*E. coli* and *E. histolytica*). In 1954, The International Commission on Zoological Nomenclature validated the name *Entamoeba* as the generic name referring to all the human amoebae having the characteristics of *Endamoeba* including the species *E. coli, E. histolytica, Entamoeba hartmanni* and *Entamoeba gingivalis.* The genus *Entamoeba* has three distinct groups according to the number of nuclei in the mature cysts being 8, 4 or 1. There are 8 nuclei in *E. coli*, 4 in *E. histolytica*, *Entamoeba dispar,* and *E. hartmanni*, and 1 in *E. polecki*. *Entamoeba gingivalis* has no encysted stage (Belding 1965). Except for the *E. gingivalis*, parasitic amoebae inhabit the

lumen of the large intestines of mammals and probably, each species has distinct strains adapted to specific hosts (Sargeaunt and Williams, 1979).

1.2.1.1.1 *Entamoeba histolytica*

Entamoeba histolytica was first described in 1875 by Losch, at St. Petersburg from the faeces of a patient with severe dysentery. Its pathogenicity in man was later demonstrated when intestinal ulceration was observed in patients with dysentery in the presence of the organism (Belding, 1965). The organism was by then not differentiated from *E. coli*. Later the difference in number of nuclei in the cysts of *E. histolytica* and *E.coli* were described and pathogenicity of *E. histolytica* confirmed in humans (Walker and Sellards, 1913). *Entamoeba histolytica* cysts measure 10-16 µm in diameter with 4 nuclei when mature and one nucleus in the precyst with a glycogen vacuole and chromatoid bodies.

In 1925, Emil Brumpt suggested there were two morphologically identical species of *E. histolytica*, one highly pathogenic and invasive (*E. histolytica*) and a non-invasive type whose presence was either asymptomatic or caused only a mild irritation naming it *E. dispar.* The idea was largely ignored until more research confirmed his findings in the 1970s (Martinéz-Palomo *et al.,* 1973). Use of isozymes of hexokinase and phosphoglucomutase enzymes demonstrated distinct patterns of invasive strains and those from carrier states (Sargeaunt *et al.,* 1982). Thereafter, development and application of monoclonal antibodies distinguished the pathogenic and non-pathogenic strains (Strachan *et al.,* 1988; Petri *et al.,* 1990; Tachibana *et al.,* 1990). Further biochemical, immunological and genetic data confirmed that the two strains

were separate species confirming them as *E. histolytica* and *E. dispar*. This led to the re-description of *E. histolytica* (Diamond and Clark, 1993; Tachibana *et al.,* 1997).

Entamoeba histolytica has a cosmopolitan distribution although incidence and morbidity are higher in the tropics. However, most information on epidemiology predates the distinction of *E. histolytica* from *E. dispar* where the latter was referred to as 'non pathogenic' *E. histolytica*. Without biochemical or immunological differentiation, it can only be presumed that the current records of *E. histolytica* may include a large proportion of *E. dispar* (Beaver *et al.,* 1984). The high prevalence of other amoebae like *E. coli* and *E. hartmanni* may also lead to misdiagnosis and over-estimation of the protozoon (Belding, 1965). A study in Philippines using PCR to differentiate the two organisms showed a 7.3% prevalence of *E. dispar* compared to 0.96% prevalence for *E. histolytica*. The reason for the prevalence disparity is not clear but *in vivo* and *in vitro* studies demonstrate differential growth with only a small proportion of *E. histolytica* outgrowing *E. dispar*, a phenomenon that will need to be investigated further (Rivera *et al.,* 1998).

In Africa, countrywide and regional surveys have recorded an *E. histolytica* serological prevalence of 14%-52% in The Gambia, 13% in Nigeria, 37% in Central African Republic (Beaver *et al.,* 1984). The prevalence of antibodies increases during the first decade of life peaking at 5-9 years, and is higher in females than males (9.3% compared to 7.1%) (Martinez-Palomo and Cantellano, 1998). In Kenya, parasite prevalence ranging from 2.5% to 13.6% were reported in different regions of the country (Chunge *et al.,* 1985; Chunge *et al.,* 1991a; Ashford *et al.,* 1992). One

study reported a parasite prevalence of 35% in a rural community in Machakos district in Kenya (Chunge *et al.,* 1995). However, there was no differentiation of the parasite from *E. dispar* and it is likely that a large proportion of these were due to *E. dispar*. While most of the asymptomatic carriers are probably infections with *E. dispar, E. histolytica* has been positively identified in some asymptomatic carriers. Majority of the latter progress to resolve the infections while about 10% develop amoebic colitis (Ghadirian and Jackson, 1987; Martinez-Palomo and Cantellano, 1998).

1.2.1.1.2 *Entamoeba coli*

Entamoeba coli is the most common of the amoebae found in humans. It was first identified in India in 1870 but it was not until 1913 that the organism was shown to be a distinct species from *E. histolytica* occurring as a non-pathogenic human parasite (Belding, 1965). *Entamoeba coli* has several stages in its life cycle that includes trophozoite, precyst, cysts that are excreated in feaces, and then if ingested, become metacyst and metacystic trophozoite which then develop to trophozoites in the colon or caecum. The trophozoite stage may sometimes be indistinguishable from those of *E. histolytica* measuring 15-50μm in diameter but the cysts are larger varying in size at about 10-31μm diameter. Trophozoites exhibit typical amoebic movements. They have an ectoplasm rim that appears granular while the endoplasm is more densely granular. The nucleus has a relatively thick membrane, lined with thick chromatin granules and contains a large eccentrically placed karyosome (Beaver, *et al.,* 1984).

The cyst form can survive adverse environmental conditions including moderate putrefaction and desiccation. When ingested, the encysted 8-nucleated amoebae are released in the intestines preceding the metacystic trophozoites that differentiate to become mature trophozoites. Trophozoites reside in the caecum, feeding on intestinal bacteria and dividing by binary fission (Belding, 1965).

Entamoeba coli has a worldwide distribution with high prevalence in both tropical and temperate countries correlating with lower income communities (Belding, 1965). It is easily transmissible through ingestion of relatively few cysts and its high prevalence indicates a high level of food and water contamination by faecal waste (Belding, 1965). Although parasites morphologically identical to *E. coli* have been found in other animals such as dogs and monkeys, it is unclear whether they are different organisms from those found in humans. Person to person infection either directly or via contaminated food is almost the exclusive form of transmission for this parasite (Beaver *et al.,* 1984). There is little if any protective immune development in hosts with age and infection and re-infection continues for life (Martinez-Palomo and Cantellano, 1998).

1.2.1.1.3 Other Intestinal Amoebae

There are other amoebae residing in the large intestines of humans but with no apparent pathology to the host. These include *E. hartmanni, E. nana,* and *I. buetschlii. Entamoeba hartmanni* cysts have four nuclei but are distinguishable from *E. histolytica* on the basis of a small cyst diameter of $<10\mu m$. Physiologically however, *E. hartmanni* differs from the former in that it is neither hematophagous

nor histophagous (Belding, 1965). Moreover, it can be differentiated from *E. histolytica* by serological techniques (Beaver *et al.,* 1984). Isoenzyme and molecular techniques have provided more data confirming *E. hartmanni* as a distinct species (Neal, 1966; Martinez-Palomo and Cantellano, 1998). Although *E. hartmanni* is non-pathogenic it has epidemiological significance since most laboratories do not routinely measure the cyst diameter hence it may be misidentified as *E. histolytica*.

Endolimax nana is also a common intestinal commensal infecting humans, primates and pigs. Like the other non-pathogenic amoebae, it is an important to differentiate the trophozoites from those of the clinically significant *E. histolytica*. Mature cysts measure between 6-8μm in diameter, have four nuclei and a distinct refractile wall measuring 80 nm thick. Trophozoites are smaller than those of other amoebae measuring 6-15 μm, with only one nucleus (Beaver, 1984).

Iodamoeba buetschlii is another of the non-pathogenic amoeba in the large intestine of humans. It is the most common amoeba in pigs, which could be the original natural hosts. Trophozoites vary greatly in size from 6-20μm in diameter. The cytoplasm has a large glycogen mass, which stains distinctively with iodine. Cysts are more ovoid than circular and are about 8-15μm in diameter.

1.2.1.2 Trichomonadida

There are two common intestinal parasites of this order, *Dientamoeba fragilis* and *Trichomonas hominis*. *D. fragilis* was first considered an amoeba but ultra-structural studies clarified its taxonomy, as a trichomonad flagellate (Camp *et al.,* 1974). It is now clear the two parasites belong to the phylum Parabasalia (Table 1.2).

Morphologically, they have a parabasal body equivalent to the Golgi body, and no mitochondria. Only the trophozoite stage of *D. fragilis* has been identified which is 6-12μm in diameter containing two nuclei. It was initially thought to be non-pathogenic but there have been reports of *D. fragilis* association with gastrointestinal disease (Sargeaunt and Williams, 1979). A study in Oman on incidence of *D. fragilis* in faecal samples submitted for routine microbiological analysis found a prevalence of 5.1% and significantly, 83% of patients with pure *D. fragilis* infection had intermittent abdominal pain lasting for up to a month over a two year period (Windsor *et al.,* 1998).

1.2.1.2.1 *Trichomonas hominis*

Only the trophozoite stage of *T. hominis* has been identified. It has a cosmopolitan distribution and is second to *G. lamblia* in prevalence of intestinal flagellates (Beaver *et al.,* 1984). There is no evidence of direct pathology due to *T. hominis.* However, it is more commonly identified in diarrhoeic stools and its presence suggest unnatural conditions in the intestines that signify enteric disturbances that might be due to other etiologies. Diagnosis is by the recognition of the characteristic wave-like movements of the undulating membrane and the spike-like posterior projection in semi-formed stools (Beaver *et al.,* 1984).

1.2.1.3 Flagellates

Intestinal flagellates have traditionally been classified in the phylum Sarcomastigophora and the subphylum Mastigophora. Currently the flagellates have been re-classified into another kingdom, Archezoa under the phylum Metamonada

(Table 1.2). This grouping separates *Giardia*, *Enteromonas* and *Chilomastix* from the trichomonads, which include *Dientamoeba* and *Trichomonas* (Cox, 1998). Again, the issue is between traditional classification and new evidence in morphology, and molecular techniques that show new forms of relatedness among the organisms.

1.2.1.3.1 *Giardia lamblia*

Giardia was probably first described by Leeuwenhoek in 1681 identifying trophozoites from his stool; it was Lambl who first described the trophozoites in 1859 under the name *Cercomonas intestinalis* (Belding 1965). In 1879 Grassi recognised the cysts, which were first thought to be coccidian, associating them with the trophozoite stage. Stiles, later assigned the name *Giardia lamblia* to the human species in 1915 and thereafter the accurate morphology was described in 1921 (Belding, 1965).

The classification and nomenclature of *Giardia* species is still unclear and continue to be debated in spite of the use of various morphological features and host specificity. Filice (1952) categorised three groups that included *G. agilis* isolated from amphibians, *G. muris* from rodents and birds, *intestinalis* infecting mammals (including human) and *G. duodenalis* from birds and reptiles (Filice, 1952). Both *intestinalis* and *lamblia* continue to be used to refer to the species infecting humans and other mammals while some researchers feel *duodenalis* is the correct taxonomic term (Beaver, 1984; Meyer, 1990). Perhaps the most important issue is consistence of the use of either terms by the clinician and corresponding laboratories (Meyer *et al.*, 1990). In their listing of references of parasites of the *Homo sapiens*, Ashford and

Crewe (1998) point out that the name *G. lamblia* has no taxonomic validity and *G. intestinalis* is the correct species name for the parasite infecting humans.

The trophozoites are 10-20µm in length and 5-15µm in width and have a characteristically pear-shape with four pairs of flagella, two nuclei, two axonemes and two median bodies. Cysts are oval-shaped measuring 11-14µm in length and 7-10µm in width, containing 4 nuclei, axonemes and median bodies (Garcia, 1998).

Transmission is through ingestion of cysts either directly or indirectly through food or contaminated water (Bella *et al.,* 1998; Garcia *et al.,* 1998). As few as 10 *G. lamblia* cysts can result to an acute infection in humans (Garcia *et al.*, 1998). The low infectious dose raises the potential risk of waterborne outbreaks attributed to *G. lamblia* associated with failed municipal water treatment (Shaw *et al.,* 1977; Dykes *et al.,* 1980; Craun 1990; Bella *et al.,* 1998; Robertson *et al.,* 2000). While it is the most commonly diagnosed flagellate from the intestinal tract, susceptibility appears to be influenced by sex, age, environmental conditions, socio-economic conditions, occupation, nutrition and overall immune status (Bennett, 1976; Meyer and Jarroll, 1980; Anand, 1983; Islam, 1990; Ahmad 1991; Garcia, 1998).

Giardia lamblia is also suspected to be a zoonosis. High rates of infections are seen in campers and hikers drinking stream water. Wildlife is suspected to be potential reservoir sources in such incidences (Dykes *et al.,* 1980). The parasite has been identified in beavers and muskrats among wildlife and in most domestic animals including dogs, cats, sheep, cattle, pigs and goats. The organism is also a common

cause of traveller's diarrhoea infecting people visiting endemic areas. Infection rates of 2-15% in western countries have been documented in people returning from international travel (Isaac-Renton *et al.,* 1986; Gray and Rouse, 1992; Overbosch, and Ledeboer 1995).

So far, acquisition and pathogenesis of *G. lamblia* does not appear to be affected by HIV infection in humans (Brasil *et al.,* 1999; Cimerman *et al.,* 1999; Fontanet *et al.,* 2000a). While it is a common infection in HIV-infected patients, there is no clinical evidence that suggest the organism is more pathogenic in such patients compared to HIV-uninfected people (Meyer, 1990; Smith *et al.,* 1988). However its role as a waterborne cause of diarrhoea in both HIV-infected and uninfected people makes it an important high public health concern.

1.2.1.3.2 Other Intestinal Flagellates

1.2.1.3.2.1 *Chilomastix mesnili*

Chilomastix mesnili is another of the commensal protozoa in the human gut. Trophozoites are asymmetrical, pear-shaped measuring 6-20µm in length and 3-10µm in width. The cysts are small, lemon shaped, measuring 7-10µm in length by 4-6µm in width. The organism lives in the lumen of the caecum feeding on enteric bacteria. Trophozoites are found in loose stool while only cysts are present in the formed stools. A study in Kenya showed significant association between the presence of C. *mesnili* trophozoites and loose/watery stools in 8.4% of the cases in children, suggesting association with gut pathology (Chunge *et al.,* 1991a). However, the link to pathology was not conclusive as the presence of diarrhea (from different causes) is

associated with an increased presense of non-pathogenic protozoa due to increased gut motility. Prevalence of *C. mesnili* ranges from about 1-10% depending on age and population surveyed. Transmission, as in the other protozoa is direct or indirect via the faecal-oral route (Beaver *et al.,* 1984).

1.2.2 Sporozoa

The phylum contains the heterogeneous group of all apicomplexan spore forming single cell organisms with the exclusion of *Perkinsus* and Microsporidia which have since been re-classified into different phyla (Cox, 1998). The human intestinal parasites included in this phylum are the coccidia that comprise *Isospora, Sarcocystis, Toxoplasma, Cyclospora* and *Cryptosporidium.* Some of these parasites have a monoxenous life cycle that is completed in one host such as *Cryptosporidium* while in others like *Toxoplasma* and *Sarcocystis*, the reproductive cycle is completed in two different hosts (Dubey *et al.,* 1990). Reproduction is in two stages within the epithelial cells of the gut that include an asexual stage (schizogony) that results in the amplification of the parasites numbers. The sexual stages (gametogony) result in the production of the oocysts, which are the infective/dispersal stage of the parasites (Beaver, 1984). Transmission is mainly person to person either direct or indirect via the faecal-oral route, while some like *Cryptosporidium* and *Toxoplasma* may be zoonotic. Dispersion of the parasites is via oocysts that can survive harsh conditions in the environment and infect a wide range of hosts including mammals, reptiles, fish and birds.

For long, coccidian parasites were largely considered as animal parasites with the exception of *Toxoplasma*. Lately, however, their association with HIV/AIDS has drawn attention to their role as significant aetiologies of opportunistic infections (Goodgame, 1996). Besides, *Cryptosporidium* and *Cyclospora* are major water and foodborne pathogens in both immunocompetent and immunosuppressed persons worldwide (Fleming, 1990; Fleming *et al.,* 1998).

1.2.3 Intestinal Nematodes

Human infection with *Ascaris lumbricoides* has been reported in almost all countries of the world with approximately 1.4 billion people estimated to be infected, with at least 1 million showing clinical symptoms (Crompton *et al.,* 1999; WHO, 1999). Over 1 billion people are estimated to be infected with hookworms and 1.1 billion with *T. trichiura.* Conservative estimates show approximately 20,000 and 50,000 deaths annually are attributed to *Ascaris* and hookworm respectively, annually (WHO, 1999).

Though morbidity depends on intensity of infection, low infections in young children may result in subtle chronic effects such as retarded physical growth and impaired cognitive skills, low participation in education and consequent poor economic performance (Bundy *et al.,* 1987; Bundy and Medley, 1992; Evans and Jamison, 1994). The parasites cause malabsorption, blood and protein losses that exacerbate any existing malnutrition conditions common in communities living in most endemic areas. Consequently, all intestinal nematodes are considered pathogenic (Crompton and Nesheim, 1976; Stephenson *et al.,* 1980).

Ascaris lumbricoides and *T. trichiura* infections are acquired early in life with high infections rates in the 5-15 years age bracket while infection with hookworms peak in adolescence remaining elevated into adulthood (WHO, 1981; Bundy and Cooper, 1987; WHO, 1987). In endemic areas, morbidity due to nematodes depends on intensity of infection, with over 60% of the worm loads occurring in 15% of the infected population (Bundy *et al.,* 1988; Bundy and Cooper 1988). Recent studies indicate a small number of individuals in a community will have heavy infections, have higher multiple infections with other parasites and are more likely to be re-infected (Anderson and Medley, 1985; Bundy and Cooper 1988; Howard *et al.,* 2001). The mechanisms that determine this aggregation or those that determine different durations of infections in the more susceptible people are not clear.

Eggs of *A. lumbricoides, T. trichiura* and hookworms are, perhaps, the most recognisable although they are usually indistinguishable between the species of each genus. Most diagnostic laboratories are able to identify the eggs or larvae of these parasites due to their distinct size, colour and characteristic shapes (Beaver, 1984). *A. lumbricoides* eggs are shed either fertilised or unfertilised and measure approximately 50-70μm x 40-50μm. The fertilised eggs have a cortical outer layer with uneven deposits of muco-polysaccharide (Belding 1965). *T. trichiura* eggs are brown in colour, lemon shaped with a plug-like structure at each end and measure 57-58μm x 26-30μm. Those of hookworms are barrel-shaped measuring 60 x 75μm x 36-40μm. The eggs of two species *Ancylostoma duodenale* and *Necator americanus* are morphologically indistinguishable.

1.3 Transmission mechanisms of intestinal parasites

1.3.1 Introduction

Epidemiology refers to the study of patterns of infection and associated diseases within populations or defined communities (Anderson, 1998). It relies on accurate surveillance and measures of infection and diseases by involving multiple disciplines in population biology, immunology, molecular biology, and specific fields of study such as parasitology. Lately, there has been more recognition of the dynamism of the host-parasite interactions in the determination of the epidemiology of infections. Observed patterns of infections are determined by the interplay between the host and the parasite with regard to susceptibility, course of infection, and rate of transmission. As a result, these interactions determine the spread, persistence and evolution of all infectious agents (Anderson RM, 1982; Dobson and Roberts, 1994).

Patterns of infection and disease development in human communities are determined by various factors, from individual susceptibility, acquisition, and duration of infections, parasite clearance and duration of acquired immunity (Holmes, 1987; Holmes, 1990). In turn, person to person transmission is influenced by their behaviour, social, environmental and demographic factors (Jose *et al.,* 1997; Anderson, 1998). Basic understanding of these factors provide insight into host parasite interactions, enhancing formulation of feasible control measures aimed at interruption and reduction of transmission and possible eradication of infections (Anderson, 1998).

1.3.2 Transmission Patterns

When a host population is exposed to parasites such as protozoa, the degree of colonisation, establishment and consequent dispersion of the invading infectious agent depends on the outcome of the subsequent host/parasite interaction (Holmes, 1987; Holmes; 1990). This contact results in any of the different states of infection among the hosts. At any one time there will be those who are susceptible and acquiring the infection, latent infectious (infected and not yet transmitting), infected and infectious (actively transmitting), and immune individuals (Anderson RM, 1982; Bundy *et al.,* 1987). These are valid where the rate of infection is assumed to vary only because of the hosts' genetic heterogeneity, behaviour or environmental differences. The different states of this host/parasite interaction are continuously at play and determine the transmission, development of a disease, and epidemiologically manifested by prevalence, incidence and intensity of infections (Grenfell and Anderson, 1985; Anderson, 1998).

1.3.3 Endemicity

Endemic disease is defined as one that is usually present in a given geographical area or population group at relatively low but constant rate, in comparison with other areas or populations (Beaglehole *et al.,* 1993). Intestinal parasites are endemic in many tropical and subtropical areas and generally appear to remain stable with only small seasonal variations, in specific populations in spite of environmental changes or control measures (Anderson and Gordon, 1982; Anderson RM, 1982). In the absence of density-dependent controlling factors, parasite populations would grow exponentially but hosts' resource limitations and intrinsic parasite factors regulate

their growth thus maintaining endemicity (Ozeretskovskaya, 1982; Stock and Holmes, 1988; Heesterbeek and Roberts 1995).

When a parasite is endemic, each primary infection produces on average, one new case. Endemicity is determined by density-dependent factors such as physical niche, level of acquired immunity within the host, age of host, and other environmental factors (Anderson RM, 1982). These in turn influence transmission, while social-economic factors or hosts' age may predispose infection or increase acquisition risks. For parasites with the faecal/oral route of transmission, environmental contamination and the hosts' behaviour should greatly influence transmission and acquisition. However, some studies suggest that in humans, exposure may not necessarily fall with age as would be expected. This is observed in the occurrence of some non-pathogenic amoebae (*E. coli, Endolimax nana*) and *B. hominis* that show no decline in older people indicating there is no reduction in exposure to faecal-oral contamination (Ashford and Atkinson, 1992). Duration of infection or perpetual re-infection rather than reduced exposure is perhaps more significant as a factor determining endemicity. However, only few studies have investigated duration of infections for intestinal parasites in specific communities (Farthing *et al.,* 1986; Gilman *et al.,* 1988; Sullivan *et al.,* 1988; Chunge *et al.,* 1991a; Chunge *et al.,* 1991b; Ashford *et al.,* 1992). Most of these studies assessed the duration of infection for giardiasis in children using different assessment methods. The method most commonly used is the follow-up examination of daily stool sampling, recording periods between infected and parasite free episodes (Farthing *et al.,* 1986; Gilman *et al.,* 1988; Sullivan *et al.,* 1988).

1.3.4 Parasite Aggregation

Parasite aggregation refers to the extent in which parasites use the available hosts unevenly, leaving most uninfected and crowding in a few (Anderson and Gordon, 1982; Anderson, 1998; Poulin, 1993; Poulin, 1996). This continuous susceptibility and repeated infections of most people living in endemic areas occur despite abundant evidence of immune recognition and response, and without corresponding development of morbidity (Maizels *et al.,* 1993; Palmer *et al.,* 2001). In a smaller proportion of the population, however, very high parasite densities develop and may result in the overwhelming of the immune reaction, disease development and sometimes death (Jones *et al.,* 1990).

The observed differences in parasite aggregation may reflect differences in innate susceptibility to infection or inability to mount effective immunological responses (Befus *et al.,* 1986; Wakelin, *et al.,* 1996; Garside *et al.,* 2000; Palmer *et al.,* 2001). Studies on cellular responses to parasite infection suggest that early exposure to infection in infancy (or earlier via maternal antibodies) may, in part, determine predisposition to heavy or light infections (Schweitzer and Anderson, 1992; Anderson, 1998). Results showed that high exposure generated tolerance with elevated Th2 responses seen in heavy helminths load (Bentwich *et al.,* 1999).

Aggregation of parasites in humans has epidemiological implications in terms of defining control measures (Guyatt and Bundy, 1991; Bundy and Medley, 1992). By occurring together in higher densities, the sexually reproducing parasites are more

likely to reproduce and enhance transmission (Guyatt *et al.,* 1990). It also increases the net-regulatory impact on parasite establishment, survival and reproduction sensitive to constraints impacted by the hosts' immune responses (Bundy and Medley, 1992; Palmer *et al.,* 2001). This has led to the debate on the significance of anti-helminthic vaccines for mass application due to the purported benefits on protection of individual disease development versus the overall negative effect on whole communities that are sustained by perpetual low level infections (Bundy, 1997). Parasite aggregation mechanisms indicate that the search for vaccines should be targeted on only those least able to develop acquired immunity (Wakelin, 1997; Anderson, 1998; Barreto *et al.,* 2000; Knox, 2000; Vercruysse *et al.,* 2001).

1.3.5 Multiple Parasite Infections and Parasite Associations

Understanding the occurrence of polyparasitism is important due to parasites' synergistic or antagonistic role on their interaction with the hosts that, in turn, influence individual or multiple parasites' endemicity or susceptibility to other infections. While many intestinal parasites occur together due to environmental or ecological coincidence, some studies suggest in some instances, there is direct interaction between parasites presence or outcome of the co-infectious agent in the hosts. *Ascaris lumbricoides* has been beneficially associated with protection from cerebral malaria (Nasher *et al.,* 2000). Another study in the western Cape (South Africa) demonstrated a positive correlation between the high prevalence of *A. lumbricoides* and *T. trichiura* infection to high tuberculosis (TB) incidence in the area (Beyers *et al.,* 1996; Warren *et al.,* 1996). The two helminths have been shown to enhance Th2 responses with high non-specific IgE that are able to activate latent

TB and induce expression of HIV co-receptors (Beyers *et al.,* 1996; Bentwich *et al.,* 1999; Bundy *et al.,* 2000). Such an association would be highly significant in devising control measures as these parasites are most common in areas with the highest incidence, morbidity and mortality of HIV and TB (Bentwich, 2000; Bentwich *et al.,* 2000; Borkow *et al.,* 2000).

Parasite associations appear to be through selective mechanisms involving host and environmental factors that determine the extent and distribution of parasites in the population (Buck *et al.,* 1978I). A stratified survey in Kilifi, Kenya showed a strong host/parasite association at the community level but less so within individual age groups or within villages (Ashford *et al.,* 1992; Ashford *et al.,* 1993). These studies demonstrated the presence of 'wormy' villages as opposed to 'wormy' individuals. While parasite associations were statistically significant, most were due to environmental heterogeneity and epidemiological coincidence rather than parasites influencing the presence of each other either positively or negatively (Ashford *et al.,* 1992).

Another study in a fishing community in Southern India showed a positive correlation between heavy ascariasis and multiple infections with *T. trichiura* and *Enterobius vermicularis.* The results suggested a predisposition for some individuals to multiple infections either through environmental or genetic factors (Elkins *et al.,* 1986; Haswell-Elkins *et al.,* 1987). A meta-analysis of surveys that included over 215,000 individuals demonstrated a significant association of infections with geohelminths especially *A. lumbricoides, T. trichiura* and hookworms in most areas

where they were endemic (Howard *et al.,* 2001). The study also highlighted the significant geographical heterogeneity between associations, hence the need to investigate specific communities in defined geographical areas. The occurrence of parasite associations together with the concept of parasite aggregation supports the concept of selective chemotherapy targeting the 'high risk' individuals as opposed to indiscriminate mass chemotherapy (Haswell-Elkins *et al.,* 1987, Elkins *et al.,* 1986).

Another study by Hall and others, (1992) showed that in a population living in an endemic area, heavy parasite clustering shifted among individuals. A three time (six months apart) follow-up study of infection with helminths, showed that 60% of the people belonged to the heavy clustering group at least once within this period. This contradicted the theory of predisposition among individuals that advocate targeted chemotherapy (Hall *et al.,* 1992; Nelson, 1992). Different intrinsic host factors including genetic predisposition have been shown to determine the degree of parasite aggregation, but the role of the host in inter-parasite association is less clear (Anderson and Gordon, 1982). Long term prospective studies on incidence and parasite interactions both in individuals and at the community level would elucidate some of the mechanisms that play a part in determining the prevailing parasite associations and aggregation (Bundy, 1991).

1.4 Intestinal parasites in Kenya

There have been numerous studies on intestinal parasites in Kenya documenting infections, treatment and control methods over the years as summarized in a retrospective review by Chunge and others, (1985). The review covered most surveys

and government records on intestinal parasites in Kenya between 1900-1980. Analysis of the surveys demonstrated that parasite prevalence had remained unchanged since the beginning of 1900s despite the social economic developments in the country (Chunge, *et al.,* 1985). Prevalence follows the geographical/climatic zones that characterize the country, and which determine parasites occurrence, transmission and dispersion.

Early studies dwelt mainly on helminths with little or no reference to intestinal protozoa (Chunge *et al.,* 1985). More recent surveys show protozoa are highly prevalent with infection rates of 80%. Prevalence is lower in children than adults and higher in females than males (Chunge *et al.,* 1991a; Chunge *et al.,* 1995; Saidi, *et al.,* 1997; Ogutu *et al.,* 1998). Most intestinal infestations are polyparasitic with different parasites showing endemicity in specific ecological zones favourable to their transmission (Chunge *et al.*, 1985; Peltola *et al.*, 1988; Ashford *et al.*, 1992).

1.4.1 Intestinal Parasites in School Children

Surveys on school children have been ongoing in Kenya for long with some records from as early as 1928. In that survey, feacal samples from a total of 142 school children in Nairobi were examined. The results revealed 62.7% had *A. lumbricoides* while 22.5% had hookworms while a small proportion (1.4%) had *Taenia* parasites (Philip and Maclennan, 1928). The first major survey of over 40,000 school children within Nairobi and surrounding regions showed a hookworm prevalence of 9.7% and 8.2% for *A. lumbricoides* (Roberts, 1949).

In western Kenya, other studies of school children recorded prevalence of 31%, and 4% *A. lumbricoides* and hookworm respectively (Diesfeld, 1969). Prevalence of the same parasites was higher among younger children in the same area. A study among 287 infants (0-4 years) showed a prevalence of 18% hookworm and 22% *A. lumbricoides* in western Kenya (Gondi-Awuor, 1974). This was almost similar to the prevalence recorded among school going children aged 5-9 yrs who showed prevalence of 15% hookworm and 38% *A. lumbricoides* respectively (Gondi-Awuor, 1974). These surveys did not include any records of the prevalence of protozoa parasites at the time.

Surveys done in the 1980's assessed the infection rates with both intestinal helminths and protozoa while some related the infections to their effects on growth and nutrition in children and/or the efficacy of various treatments (Wijers *et al.,* 1972; Stephenson *et al.,* 1979; Stephenson, 1980; Stephenson, *et al.,* 1989). The studies highlighted the association of geohelminths especially *A. lumbricoides* with protein and energy malnutrition in children. Results showed significant improvements of growth as a result of reduction of parasite intensities below clinical significance and speculated that this would lead to a reduction in environmental contamination, an effective long term parasite control strategy (Stephenson, 1980)

1.4.2 Intestinal Parasites in Rural Communities

Surveys of rural communities in Kenya suggest infection rates with intestilan parasites are common (Chunge *et al*., 1985). In a study on a rural community in central Kenya, comprising 56 families and 461 individuals, 77% of all stools

examined were positive for at least one parasite (Chunge *et al.,* 1991a). Prevalence of protozoa increased with age with *G. lamblia* peaking among 0-4 year olds. Infection was higher in females than males except for *A. lumbricoides* that was higher in men. In the study, duration of infection for 11 parasites was analysed with *Hymenolepis nana* showing the longest duration of 237 +/- standard deviation (SD) 151.4 days, while *T. hominis* showed the shortest duration of 41+/- SD 0.4 days. *Giardia lamblia* had a duration of infection of 131.7 +/- 26.7 days, while *Ascaris* had a duration of infection of 120.4 +/- SD 44 days (Chunge *et al.,* 1991a). This was the first study to estimate duration of infections in specific parasites in Kenya. The estimates were means for three consecutive surveys done 184, 120, 153 days apart, pooling the data from both children and adults. In the analysis, the long unequal durations between surveys could have contributed to the large values of the standard deviations observed.

In another earlier study, faecal samples were obtained from four regions of Kenya that included the humid coastal region, the cool highlands, the equatorial region and the marginal arid areas of the north east (Hall *et al.,* 1982). This was a study on intestinal helminths among men working on road construction. In all, 70% of the people examined had intestinal helminths with hookworm as the most common infection with a prevalence of 74% at the coast, 39.6% in the highlands, 25% and 14.3% in the lake Victoria basin and arid north east respectively (Hall *et al.,* 1982).

Prevalence of protozoa cysts was 39.7% in the highlands, 24.4% at the coastal region, 26.5% in the Lake basin and 37.7% in the arid marginal areas. Variation of prevalence between local communities of road construction workers was common.

However, the patterns perhaps reflect typical parasite burden among rural people in most parts of Kenya (Arnold *et al.,* 1978; Hall *et al.,* 1982). However, interpretation of these results is difficult as construction road workers would be recruited from many different parts of the country while their continuous movement would lead to persistent exposure and infections of different parasites depending on the location.

1.5 Diagnosis of intestinal parasites

Identification of intestinal parasites is done either for individuals in clinical and research laboratories or for epidemiological surveys where whole communities are examined. Most diagnosis for individual specimens in laboratories is undertaken using microscopy on direct fresh smears and/or concentrated faecal samples where the main interest is in ova/cysts or trophozoites of any parasites present. For epidemiological surveys using microscopy, faecal samples are collected and sometimes have to be transported long distances to research laboratories. Thus the process requires not only collection but also preservation and transportation of the sample before examination is done (Beaver *et al.,* 1984).

Once in the laboratory, appropriate examination method is selected to enable optimum detection and identification of the intestinal parasites. Efficient diagnosis is therefore aimed at identifying the various stages of each parasite in order to differentiate and identify them accordingly (Beaver, 1984).

1.5.1 Collection of Specimen

Samples must be collected in clean, labelled containers without contamination with extrinsic material such as urine or soil, and covered to avoid desiccation. Gross examination of the stool will indicate the colour, smell and consistency, which may aid in diagnosis. Formed stools are not likely to harbour trophozoites and can be kept overnight or at 4^{0}C without preservatives, while semi-formed or diarrhoeic stools must be examined or preserved immediately for identification of trophozoites. However, in some instances, trophozoites present in formed stools are identified when permanent stains such as Trichrome or Iron haematoxylin are used to enhance sensitivity.

1.5.2 Preservation

Epidemiological surveys require the collection of large numbers of faecal samples where it may not be possible to examine all the samples in the field location. Preservatives are generally selected depending on various factors including the ease of preparation, handling and any intended permanent smear to be used. Several formalin based preservatives such as 4% or 10% formal saline, or sodium acetate formalin (SAF) are the most commonly used. Formalin based preservatives are best where faecal concentration methods are intended. Others are polyvinyl alcohol (PVA), Schaudinn's solution, and merthiolate-iodine-formaldehyde (MIF). These are more appropriate when permanent stained smears are intended but are less efficient for concentration methods (Beaver, 1984). Whatever preservative is used, the volume must be adequate so that the faecal sample is completely emulsified and no large particles are left unsuspended.

1.5.3 Examination

Various methods of stool examination are used routinely. The most common is the direct method of a saline or iodine stained faecal smear. Direct examination or stained smear is also done on the resulting pellet where initial concentration method is used. The number of stools examined and the method of examination remains the discretion of the investigator as there is no one 'gold standard'. However, direct examination (of fresh faecal preparation or from concentration) coupled with a permanent stained smear is the most reliable to make definitive diagnosis as internal features of the parasites can be discerned (Beaver, 1984).

There are several techniques that have been devised to increase the sensitivity of direct examination. Nazer and others, (1993) showed at least three stool specimens were required to have a conclusive diagnosis. In their study, a first examination of stool samples yield 58.3% positives, a second examination of the same samples identified another 20.6% while a third examination on the same specimens yielded an additional 21.1% positives. Thus, in their study a single direct examination of stool sample may only yield approximately 60% positive results while almost 40% are false negative (Nazer *et al.,* 1993).

Similar results on the usefulness of 3 consecutive stool samples were obtained in a study in California (Hiatt *et al.,* 1995). In the study, 3 examinations increased the yield for *E. histolytica* by 22.7% (CI, 11.8-33.5%), 11.3% (CI, 6.9-81.8%) for *G.*

lamblia, and 31.1% (17.7-54.4%) for *D. fragilis*. These results show that perhaps a third of positive samples may be missed by the use of a single stool examination.

Some researchers have recommended that only examination of the concentrate and permanent stained smear should be carried out in routine laboratories, questioning the usefulness of direct wet mount (Estevez and Levine, 1985). Whichever method is chosen however, reliability of the results depends on the examiners experience and how well the selected technique is applied (Kaminsky, 1978; Engels *et al.,* 1996). This is determined by incorporating quality control in routine examinations for individual parasite identification or epidemiological surveys (Ayala and de Sanchez, 1974). The method chosen will depend on clinical and epidemiological data available in the area, the costs of the tests, minimum answer time, and methods of interpretation of the results obtained (McMillan and McNeillage, 1984; Aucott and Ravdin, 1993).

1.6 General Objective:

The general objectives of our study were:

- To describe the patterns of infection of intestinal parasites in relation to non clinical HIV-infection in a rural community in Western Kenya
- To determine the prevalence of *Cryptosporidium* parasites from this community and identify the precise species and genotypes isolated

1.6.1 Specific Objectives

The specific objectives of the study were to:

1. Outline the prevalence of intestinal parasites in mothers and children in relation to their different villages of residence
2. Investigate the prevalence of intestinal parasites in mothers and children with reference to their HIV status
3. Undertake a follow-up study of the cohort of mothers and children to investigate the incidence, clearance rates, and duration of infections in mothers and children in reference to the HIV status
4. Outline the prevalence of *Cryptosporidium* in mothers and children with or without HIV infection
5. Identify by genotyping and sequence analysis of the 18S rRNA gene, the precise species of *Cryptosporidium* organisms isolated in the study cohort and those recovered from HIV-infected and uninfected people in Malawi, Vietnam, Brazil, UK and Thailand

1.7 Justification

In spite of the concerted national, regional and personal efforts in the improvement of public health, intestinal parasites are endemic in Kenya with almost 100% life-time prevalence. Numerous epidemiology and control programs have been formulated, majority of them targeting schoolchildren. Efforts in treatment and control of parasite infections have been undertaken, but few studies have aimed at investigating the transmission mechanism of these parasites in the communities. While exposure and risk factors may be similar in many areas, there are large disparities in the prevalence

of intestinal parasites even in communities with similar socio-ecological factors. Understanding some of the transmission mechanisms for pathogenic and non-pathogenic intestinal parasites can elucidate these differences. This has become urgent with the emergence of HIV/AIDS that has exacerbated the state of infectious diseases in most communities including those in western Kenya.

Cryptosporidium is not routinely investigated in clinical laboratories, as its occurrence is still considered insignificant in Kenya. However, studies elsewhere have shown an increase in incidence in communities not only due to HIV infection but also due to increased poverty, poor sanitation, malnutrition, and perhaps increased contacts with animal reservoirs that increase risk of acquisition. While the apparent increase in incidence is evident, the species or strains infecting humans and their implication on epidemiology of cryptosporidiosis remain unknown.

The aim of our study was to investigate the state of intestinal parasites, their transmission dynamics and duration of infections in a rural community in western Kenya. The effects of HIV infection on these profiles in both mothers and children was undertaken. Further studies included the detection and genotypic analysis of *Cryptosporidium* present in the communities and compare them to others from similar communities affected by HIV in Malawi, Thailand, Brazil and Vietnam.

2.0 CHAPTER 2: EPIDEMIOLOGY OF INTESTINAL PARASITES

2.1 General Material and Methods

2.1.1 Introduction

This chapter covers the materials and methods used for the first phase of the study on epidemiology of intestinal parasites in a rural community in western Kenya.

2.1.2 Study area

The study was carried out in Kisumu and Busia areas of Western Kenya. Geographically, the area is a hot and humid lake basin with a varying hinterland. The study area included seven villages in all, divided into two groups labelled for study's logistics as Kisumu and Busia cohorts. The villages were spread in several divisions of Kisumu, Siaya and Busia districts. Three villages comprised the Kisumu cohort namely Chulaimbo, Kombewa, and Nyahera, while four villages were classified under the Busia cohort comprising of Khunyangu, Matayos in Busia District, and Usigu and Siaya, in Siaya District.

2.1.3 Geography

Kisumu District in Nyanza Province lies within Longitudes 33^0 20'E and 35^0 20'E and Latitudes 0^0 20' South and 0^0 50 South. The district is in the lowlands surrounding the Nyanza Gulf, with prominent physical features such as scarps in the north, east and south and lowland plains across the Kano regions, all part of the topography of the Great Rift Valley.

The major physical features are overhanging huge granite rocks at Kisian and the legendary Kit Mikay in Maseno Division while Lake Victoria to the west, is the other dominant feature in the region. The mean annual rainfall varies with altitude and proximity to the lake in the west or the highlands in the east, ranging from 1,280 to 1,800mm. Like the rest of Kenya, the area has two rainy seasons with the long rains occurring in April/May and the short rains in September/October. The short rains may be erratic resulting to long spells of dry season. The mean annual maximum temperatures range from 25^0C to 30^0C and the mean annual minimum temperature range from 9^0C to18^0C depending on the altitude that ranges from 1,144 m above sea level at the lake side and the low lying plains, to 1,525 m above sea level in higher areas of Maseno and Nyakach district (Source: District Development Plan; Kisumu Government of Kenya, 1997-2001). The soils are predominantly lake sediments usually sand and clay soils. To the east are the Kano plains with poorly drained dark cotton soils while in the west and north-west (the study area), the soils are granite extensions of the rocky Kakamega uplands.

The other villages (Usigu and Siaya) in Siaya District, (Matayos and Khunyangu) in Busia district are further north and north west of Kisumu. Here, rainfall is again bimodal and distribution varies with altitude being drier to the south along the shores of the lake and progressively wetter towards the hinterland in the higher altitudes in Busia. Annual rainfall ranges between 1800mm to 2000mm falling in a similar pattern to that in Kisumu district. The mean temperatures are about 21^0C with a minimum of about 15^0C and a mean maximum of about 30^0C. Humidity is relatively high but the rainfall moderates the temperature and enhances farm productivity in the

district (District Development Plan; Siaya Government of Kenya, 1997-2001). Most residents are small-scale subsistence farmers with about 2.4 to 4.0 hectare holdings supplemented by formal or informal jobs in the local town centres while others depend on the local fishing industry in the neighbouring Lake Victoria.

2.1.4 General Health and Health Infrastructure

The area is well served by government-run district hospitals (Kisumu, Siaya and Busia) with all villages having access to a divisional health centre within a distance of approximately 10Km. Among the infectious diseases, malaria is considered the leading cause of morbidity and mortality followed by the gut parasites that also have a high morbidity. A previous study on knowledge on the occurrence and transmission of gut parasites in the community is average to good especially due to the emphasis of helminths control from public health officers through the local health centres and schools (Kamunvi and Ferguson, 1993). In most areas, residents have access to piped water supply except in Kombewa, Nyahera (Kisumu district) and Usigu, (Busia). In these villages, residents fetched water from nearby streams or rivers. Lately, the communities have been affected by the human immuno-defiency virus (HIV) resulting in the development of acquired immunodeficiency syndrome (AIDS) with some areas recording the highest prevalence of HIV infection of over 30%.

2.2 Initial Cohort Background

In 1996 a study on Mother to Child Transmission (MTCT) of HIV prevention using zidovudine (AZT) was commenced in the area by scientists from the Kenya Medical Research Institute (KEMRI) with Dr D. Koech and Dr P. Tukei as the Principle Investigators. The study was funded by the KEMRI/Japanese International Co-operation Agency (JICA) collaboration research –Protocol No 419 Approved by Scientific Steering Committee, April, 1996), and was underway when we initiated our study.

Fig 2.1 Study Area in western Kenya

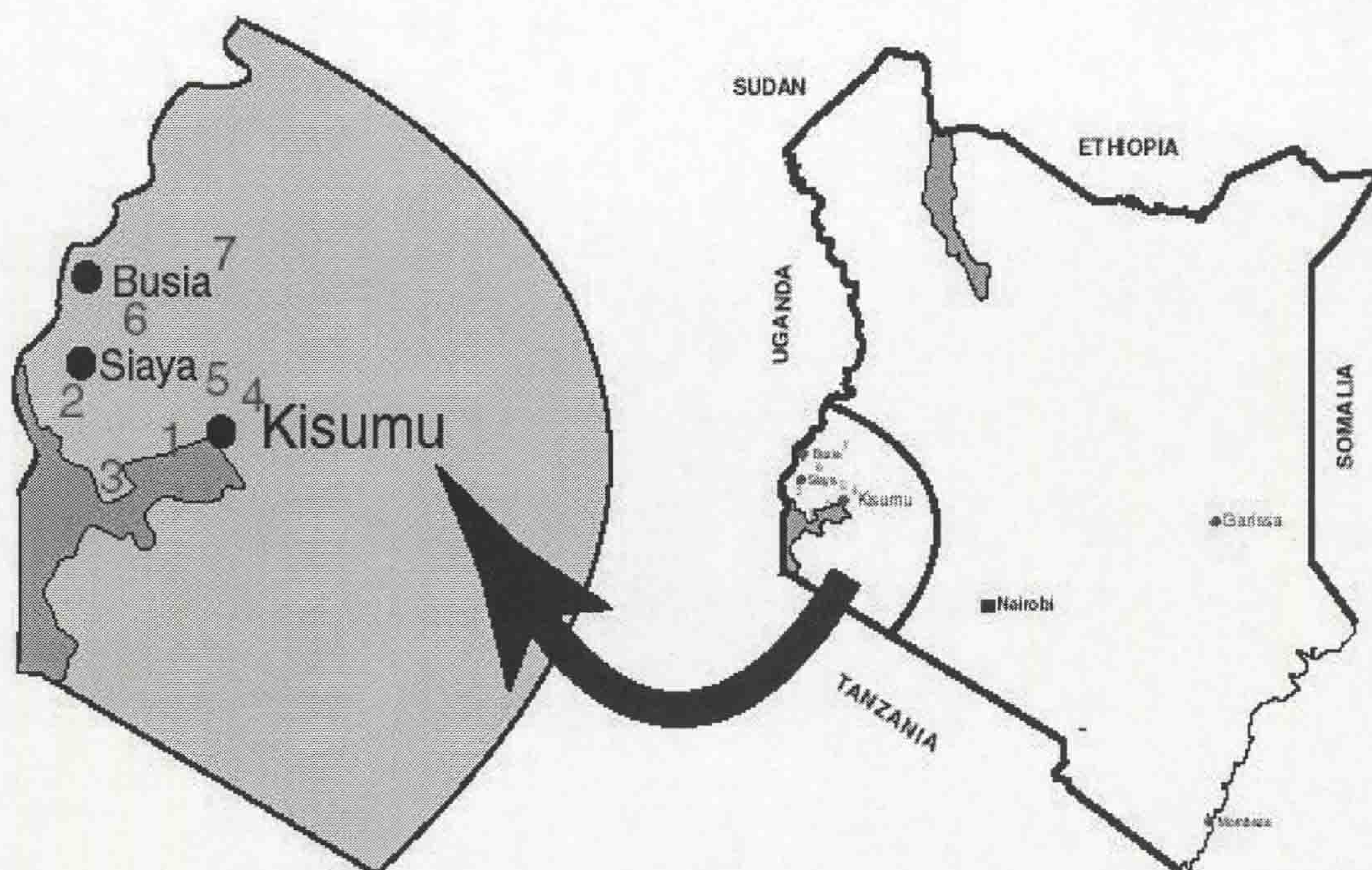


Fig 2.1 shows the study area in western Kenya. Numbers in the inset map indicate the different villages surveyed. 1-Chulaimbo, 2 –Kombewa, 3- Nyahera, in Kisumu while 4-Khunyangu, 5-Matayos, 6-Siaya and 7 – Usigu in Busia district.

The first and second phases of the MTCT study commenced in Dec 1996-1998 and 1999-2001 respectively. The overall aim of the study was to evaluate the use of a short-course regimen of prenatal zidovudine (Zdv) in the prevention of mother-to-child transmission of HIV virus in expectant mothers in rural western Kenya. A cohort of 825 pregnant women (18-22 weeks gestation; mean age, 23.2 years) from areas shown above were recruited (Fig 2.1). They were counselled and consented to enrol in the study. Expectant mothers showing clinical signs of HIV infection (AIDS) were excluded from the study, as was anybody who declined to participate. Within the MTCT study objectives, medical evaluations were undertaken on the consenting mothers at the local health centres in collaboration with local health officials. A total of 216 (26%) women were positive for anti-HIV antibodies. The new born children were followed up and were assessed clinically every 3-4 months for 2 years after birth and thereafter, were free to attend medical-check ups until the age of 5 years. Children born to HIV infected mothers testing positive by anti-HIV antibody tests at birth were presumed positive for HIV until confirmed negative at the end of the 18 months follow-up period. At this time, the child's HIV status was confirmed by both polymerase chain reaction (PCR) and serological tests as described by Songok and others (2001). The number of recruited and exiting mothers was closely monitored to maintain the initial number of participants as shown in Table 2.1

Table 2.1 Summary of Village participants enrolled in the study MTCT (June, 1999)

Cohort	Village	Mothers	Children	Totals
Kisumu	Chulaimbo	111	78	189
	Kombewa	168	101	269
	Nyahera	120	83	203
Busia	Khunyangu	72	44	116
	Matayos	127	88	215
	Siaya	93	55	148
	Usigu	147	132	279
Total		**838**	**581**	**1419**

2.3 Intestinal parasite study

Our study was a survey of intestinal parasites from a group of mothers and their children enrolled in the larger MTCT cohort study described above, within the seven villages shown in Fig. 2.1. Consent wa s sought from all mothers or guardians of the participating children after the study objectives were outlined to them (see Appendix 1).

Field sampling and data collection was done at the beginning of the survey and thereafter at 3 and 6 months; contact was maintained through field workers stationed in the villages throughout the study. Examinations were carried out within the health centres or district hospitals nearest to the respective villages. Results were communicated to the medical officers in charge and to the participants who were free to seek treatment accordingly.

2.3.1 Study Sample Selection

We selected the MTCT for our field study as it offered a rural setting and besides, the study did not include enteric parasite research, an important health concern in the area. Since the study was already underway, the framework was set to examine and

follow up the mothers and their children within a period of six months. Approximately a third of the mothers were HIV infected although not with symptomatic AIDS. The villages in the study cohort were spread over several locations with varying geographical and ecological factors but fairly similar socio-economic settings. All consenting mothers and their children were recruited in our study for the period of the survey. Participants were informed through the local health and field workers to attended the local health centres for examination and sample collection every three months, during the MTCT follow-up. From the 830 mothers enrolled in the MTCT study, approximately 500 were expected to take part in our study allowing for a 60% participation.

2.3.2 Biases

Since the MTCT study had been going on for two years previously, the mothers were well informed on health issues and were unlikely to harbour any serious illnesses as medical care and advice was provided through the local health care facilities and field workers. We were not able to take part in the initial cohort selection, as this was an ongoing study. Our field survey was limited to six months, making it difficult to organise our own cohort. Our survey was therefore biased towards parasites common in adults, as the majority of participants were mothers and those occurring in very young children of between 0-2 yrs old.

Working within the MTCT study meant that we were affected by any logistical problems that the primary study faced including the perceptions the mothers had on their expectations of the researchers. Difficulties in strict time schedules and communication failure through the respective field workers at times affected the

mothers' participation in our survey. Undertaking a followup study also meant a proportion of mothers and children would drop out of the study, events that could ultimately affect our general objectives.

2.3.3 Study Design

This was a "survey follow up study" that combined cross-sectional and cohort designs. A cross-sectional study was done on all the consenting mothers who attended the initial examination and every other survey combined with cohort study comprised of the participants who attended the follow-up surveys.

The first examination (Exam1) was done in June 1999 after initial recruitment of consenting mothers and their children. Each village was visited once during the follow up period. The second survey (Exam 2) was done at the end of September 1999, again with each village being visited once while the third and final survey (Exam 3) was carried out at the beginning of January 2000. All stool collection commenced around 10.00hr and continued up to around 15.00hr when the surveys ended. Constant contact and dialogue between the participants, field workers and the researchers was maintained between surveys in the respective villages.

In all stool collections, mothers were issued with clearly labelled stool pots. Different colours of labelling were used to distinguish the stool pots for mothers and those for the children. Direct wet mounts were examined at each of the field stations and the results communicated to the mothers. They were free to seek any medical advice based on the results given.

2.3.4 Data Analysis

All results were recorded in MS Excel (Windows 98®) spreadsheet. Information included participant's identification number, cohort and village of residence, if mother or child, and the HIV status.

Data cleaning and analysis was done in MS Excel and in SPSS (ver10.0)

Descriptive statistics including parasite prevalence, means, standard errors and confidence intervals were computed for all participants examined in relation to their village of residence, and HIV status.

Point prevalence for each parasite was expressed as

$$\text{Point prevalence} = \frac{\text{Number of persons found infected at the time of survey}}{\text{Total number of persons examined}}$$

$$\text{Period prevalence} = \frac{\text{Total number of persons found infected during the specified period}}{\text{Total number of people examined once during the period}}$$

Differences in parasite prevalence between mothers and children and by HIV status were assessed Fishers exact tests in all the Chi square (X^2) analysis due to the low number of participants especially in children. Differences in means of parasite prevalence in the seven villages were assessed using one way analysis of variance (ANOVA). Multiple parasite infections were calculated as total counts, means and standard deviations of the individual species in any one sample. This was related to the age, examination and HIV status

To assess the effect of any variable on the outcome of presence or absence of specific parasite infections, logistic regression was applied. The identified predictor variables were age, village, survey (Exam) and HIV status in the cohort. Logistic regression coefficients were used to estimate odds ratios for each of the independent variables in the model.

Crude incidence and clearance rates were calculated for the periods between any two examinations. Crude incidence measures the flow of individuals from 'free of infection' to 'infected' (Kahn and Sempos, 1989). Incidence was therefore computed for only those who were free of infection at the initial examination and thereafter became infected in the second examination. Conversely, clearance rate is defined as the proportion of the people becoming negative for the infection over all those initially infected within a defined period of time.

$$\text{Incidence} = \frac{\text{Number of those found infected at the second examination}}{\text{Total number of found uninfected at the first examination}}$$

$$\text{Clearance} = \frac{\text{Number of those found uninfected at the second examination}}{\text{Total number of those found infected at the first examination}}$$

Duration of infection was calculated using the method described by Bekessy *et al.*, (1976). This was computed from the daily transition rates as described in Appendix 3.

2.4 Faecal Sampling and Examination

2.4.1 Materials and Methods

2.4.1.1 Examination 1

The first examination was carried out in the first week of June 1999. This is the cool and dry season in the region with little farming activity. During this first encounter, mothers were briefed, in their local languages about the study, its aims and objectives and methods to be used for sample collection. All participating mothers were requested to give a signed or thumb print consent for their participation and/or that of their children. Field surveys commenced around 10.00hr local time and continued until around 15.00 hr. In this survey, a high number of mothers turned up, but many were unable to give a stool sample from themselves or their children indicating they had already relieved themselves earlier in the morning.

2.4.1.2 Examination 2

The second examination was undertaken in the last week of September and first week of October 1999, which marks the beginning of the short rains. Mothers and their children again assembled in the respective health centres or district hospitals for the MTCT follow up study. Stool pots were issued with the labels and identification numbers marked in different colours for mothers and children.

2.4.1.3 Examination 3

The third and final survey was done in the second and third week of January 2000. This is the period that is characterised by hot and dry climate in the region. Socially, it is a busy time for parents as it marks the beginning of school for most of the 6 yr old children and

the advancing of the older ones to the next grade in school. Mothers enrolled in the study again attended the health clinics on the allocated days of examination. Stool pots were distributed and they were reminded of the colour labels for their samples and those from the children.

2.5 Sample collection

Faecal sample collection was done on each of the scheduled days in the respective village.

Procedure:

Mothers were issued with a 50ml clearly labelled stool sample container, and where possible one for each child accompanying the mother. Different colours of labelling were used to help them differentiate the containers as described above. Sample containers were labelled with the identification number, mother or child label, and date of collection. All stool samples collected were placed on ice for subsequent examination and processing.

Reagents for stool sample examination

Sodium acetate formalin (SAF):	1000ml
Sodium acetate	15 gm
Glacial acetic acid	20 ml
Formalin	40 ml
Distilled water	925 ml

Potassium dichromate (Aqueous 2.5%)

2.5.1 Field Examination and Sample Shipping

Using a wooden applicator, a small portion of faecal sample was placed on a clean microscope glass slide and mixed with a drop of saline or Lugol's Iodine (10g potassium iodide dissolved in 100ml distilled water then added 5g of iodine crystals;

stored in a dark bottle). The sample was examined under low and high magnification X100 and X200. The results from the wet mounts were communicated to the mothers on the day of examination.

The remaining sample where possible, was divided into two portions with one sample fixed in sodium acetate formalin (SAF) for further analysis and the rest put in 2.5% potassium dichromate and stored at 4^0C. Where only a small sample was given, all was diluted with SAF. Samples were transported to KEMRI laboratories in Nairobi for concentration and further direct examination. At the end of the surveys, a small portion of each sample was shipped to Liverpool, UK for staining and more detailed analysis.

2.6 Laboratory Examination

Samples were examined at the KEMRI laboratories and at the LSTM using the direct wet mount method. Where necessary a second sample was prepared for staining as described below

Equipment and reagents

Frosted pre-cleaned glass slides

18 x18 mm cover slips

Applicator sticks

Normal saline (0.85% NaCl in distilled water)

Lugol's Iodine - (as described in Section 2.5.1)

Procedure:

Direct smears were prepared by mixing a small amount of the SAF-preserved faecal sample (with minimum debris) with normal saline on a clean microscope slide, and covered with a clean cover slip. Examination was done on all the areas of the cover slip under x100 and x400 magnification. In another specimen of the same sample a drop of Lugol's Iodine was used for identification of cysts. The smear was again examined at X100 and X400 for detailed parasites cysts identification. Each sample was examined at least three times using the direct method.

2.6.2 Trichrome staining

Trichrome stain preparation- (Dagnall Teaching Laboratory Manual, Liverpool School of Tropical Medicine, 1999© Adopted from WHO recommended method manual : Kokoskin E, 1993, McGill University, Canada)

Only samples whose parasite identification was uncertain with the direct smears were processed for trichrome staining. This was done according to the protocol described in Appendix 2

3.0 CHAPTER 3: RESULTS OF PARASITES PREVALENCE

3.1 Introduction

This chapter presents the results of the intestinal parasite survey. It describes the point and period prevalence observed in both HIV-infected and un-infected mothers and children. The occurrence of multiple parasite infections is examined. The risks of parasite infections are assessed. Discussion of these results is also presented.

3.2 Overall Parasite Prevalence

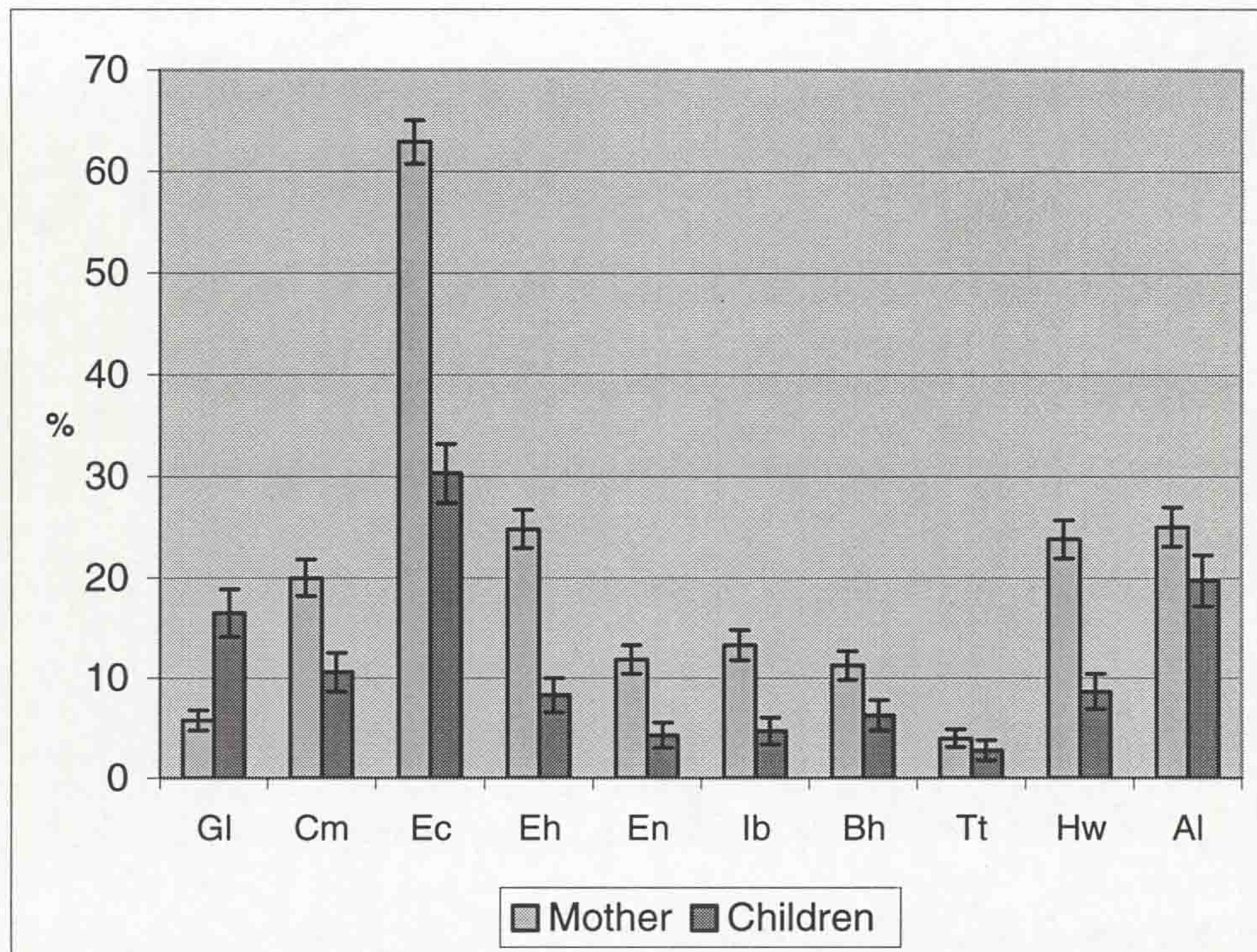
Cross-sectional surveys were done in three consecutive visits each approximately three months apart. A total of 758 stool samples were examined throughout the study period. Of those, 504 (66.5%) were from mothers and 254 (33.5%) from children. Out of the total number of mothers who gave a stool sample, 375 (74.4%) were HIV un-infected (negative) while the remaining 129 (25.6%), were HIV-infected (positive). Among the children, 196 (77.2%) samples were from HIV-negative children and 58 (22.8%) were from HIV-positive children. HIV status in mothers and children was assessed by ELISA method and confirmed by PCR as described Songok and others (2001). Children born to HIV-infected mothers showing HIV antibodies were presumed infected until confirmed as negative by PCR at the age of 18 months.

Stool examination revealed the presence of numerous intestinal parasites, the most common being *Entamoeba coli* (Ec), and *Entamoeba histolytica /E. dispar* (Eh) (since there was no biochemical distinction between *E. histolytica* and *E. dispar* any reference in our work on *E. histolytica* includes both organisms as identified by microscopy). Others were *Giardia lamblia* (Gl), *Chilomastix mesnili* (Cm),

Blastocystis hominis (Bh), *Endolimax nana* (En), and *I. buetschlii* (Ib). Among the helminths, *Ascaris lumbricoides* (Al), Hookworm species (Hw), and *Trichuris trichiura* (Tt) were the most common. Other parasites identified infrequently included *Schistosoma mansoni, Hymenolepis nana, Enterobius vermicularis*, and *Trichomonas hominis*. Parasite identification was based on morphology of cysts and trophozoites for protozoa, eggs and occasionally larvae for the nematodes. Since the overall prevalence of the least frequent parasites was less than 2%, subsequent analysis was restricted to the ten most common intestinal parasites

Among the coccidia, *Cryptosporidium* oocysts were the most common, while three cases of *Isospora belli* were identified and no case of *Cyclospora* was recorded from the cohort. The results of the coccidia are presented in Part 2 of the thesis.

Fig 3.1Overall parasite prevalence in all examinations



Bars represent Standard error of the mean

Fig 3.1 shows the overall parasite prevalence in the total samples examined throughout the study period in both mothers and children. Infections with the amoebae were the most common while *T. trichiura* was the least common. An overall *E. coli* prevalence of 62.9 % ± 4.2 Confidence Intervals (95%CI) and 30.3% CI ± 9.5 was recorded in mothers and children respectively (Confidence intervals are shown where significant differences were recorded). Among the nematodes *A. lumbricoides* and hookworm infections were generally high as expected in this region with a mean prevalence of 25% and 19.7% in mothers and children respectively. Differences in parasite prevalence between mothers and children are as shown in Table 3.1 below.

Table 3.1. Frequency of individual parasites in all the stools examined in the study

	Individual parasite prevalence (%)									
	Gl	Cm	Ec	Eh	En	Ib	Bh	Tt	Hw	Al
Total n =758	9.4	16.9	52	19.3	9.4	10.4	9.6	3.6	18.7	23.2
Mother (504	5.8	20.0	62.9	24.8	11.9	13.3	11.3	4.0	23.8	25.0
Children (254)	16.5	10.6	30.3	8.3	4.3	4.7	6.3	2.8	8.7	19.7
p value	**0.001**	**0.017**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	0.265	**0.001**	0.06

Degrees of freedom (Df) = 1, *p* values in 'bold' show parasite prevalence that were significantly different between mothers and children (Fishers exact test)

Table 3.1 shows the results of the total individual stools examined throughout the study results showed prevalence of each parasite was significantly higher in mothers than children (*p*= 0.000-0.017) except in *A. lumbricoides* (*p*=0.06) and *T. trichiura* (*p*=0.265) where no significant difference was observed. Children had significantly higher infection rates with *G. lamblia* (*p*=0.01) compared to mothers.

3.3 Parasite Prevalence in each Examination

3.3.1 Results Examination 1

In this first visit, 316 participants were able to give a faecal sample from the seven villages visited comprising of 220 mothers and 96 children. The number examined represented only about 23% compliance of the total participants enrolled in the MCTC study. Poor attendance was attributed to diverse reasons including communication breakdown between the mothers and respective field workers. This resulted in mothers sometimes failing to attend examination on specific days allotted to each village. In other cases, family chores and household activities were cited as reasons for failure to attend.

Table 3.2. Parasite prevalence in mothers by villages Exam 1

		% parasite prevalence									
Village	N	Gl	Cm	Ec	Eh	En	Ib	Bh	Tt	Hw	Al
Chulaimbo	17	5.9	11.8	70.6	29.4	5.9	17.6	17.6	0	23.5	23.5
Kombewa	29	6.9	24.1	55.2	13.8	6.9	17.2	10.3	3.4	6.9	20.7
Nyahera	43	7.0	11.6	65.1	11.6	2.3	11.6	2.3	4.7	20.9	20.9
Khunyangu	28	0	14.3	57.1	21.4	3.6	10.7	0	3.6	25.0	39.3
Matayos	48	0	14.6	64.6	20.8	4.2	12.5	12.5	2.1	27.1	22.9
Siaya	26	7.7	19.2	46.2	23.1	15.4	11.5	3.8	3.8	26.9	23.1
Usigu	29	0	24.1	55.2	27.6	0	10.3	10.3	0	17.2	48.3
Total	220	3.6	16.8	59.5	20.0	5.0	12.7	7.7	2.7	21.4	27.7
p- value		0.263	0.708	0.625	0.573	0.203	0.975	0.169	0.898	0.470	0.099

Analysis of variance one way, (p>0.05); Degrees of freedom (Df) =6

Table 3.2 shows the parasite prevalence among mothers in the seven villages in the study. Infection rate with the amoebae were highest in Chulaimbo and Matayos villages with prevalence of 70.6 %, and 64.6 % respectively. Both villages had piped water supplies. *E. histolytica* prevalence was about 20% in all the villages although there were no clinical reports of diarrhoea (corroborated with results of MTCT clinical survey). Usigu, the village closest to the lakeshore recorded the highest prevalence of *A. lumbricoides*, of 48.3 %.

The prevalence of individual parasites in the seven villages was calculated by analysis of variance one way (ANOVA). There were no significant differences between the villages in either the protozoa or the helminths infections (p = 0.099-0.975).

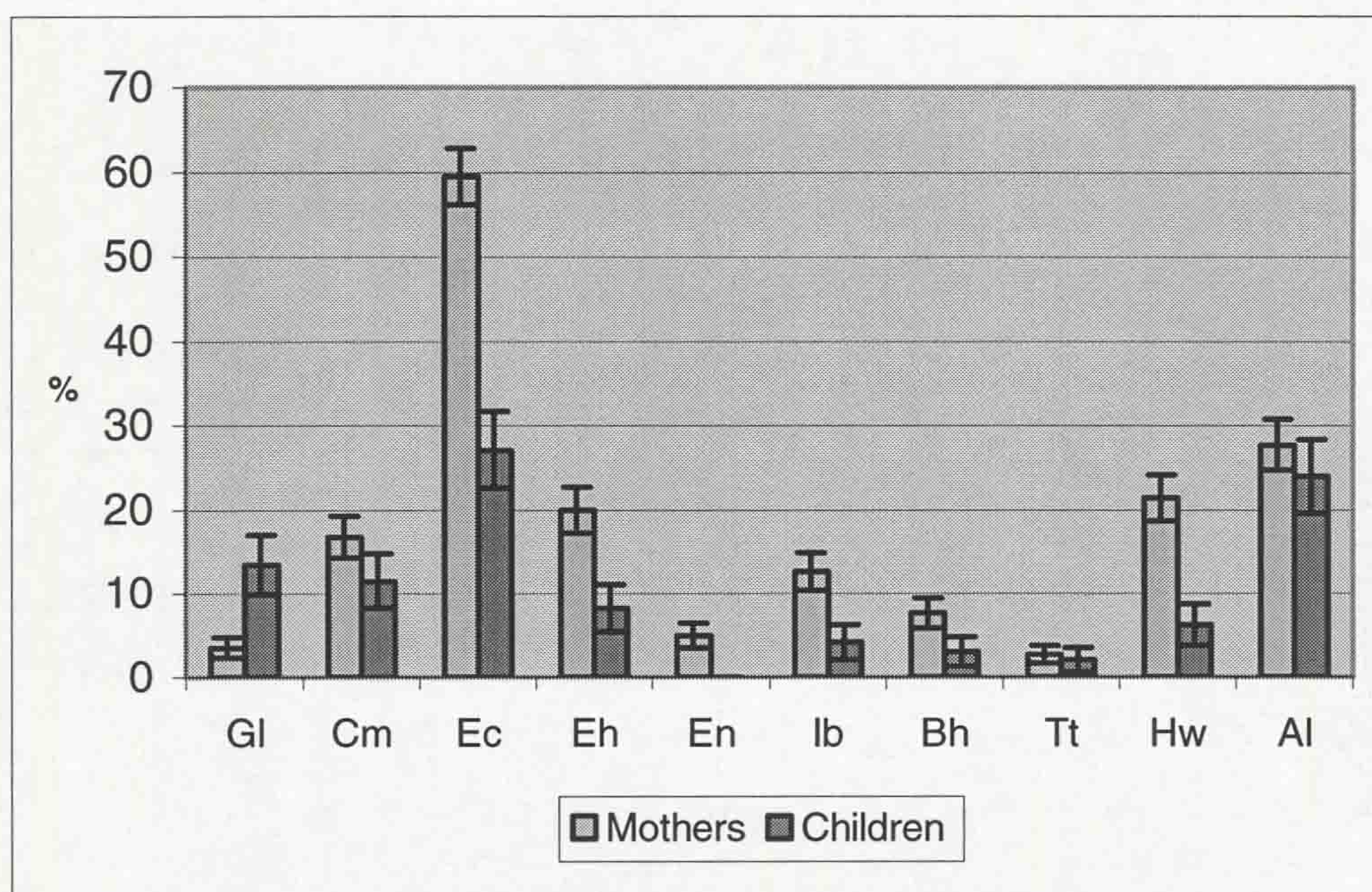
Table 3.3 Parasite prevalence in children by village Exam 1

		% parasite prevalence									
Village	n	Gl	Cm	Ec	Eh	En	Ib	Bh	Tt	Hw	Al
Chulaimbo	11	18.2	18.2	36.4	0	0	9.1	0	0	0	45.5
Kombewa	13	7.7	15.4	61.5	15.4	0	7.7	0	0	15.4	30.8
Nyahera	22	18.2	9.1	13.6	4.5	0	0	4.5	4.5	9.1	18.2
Khunyangu	15	13.3	13.3	40.0	13.3	0	13.3	0	0	6.7	33.3
Matayos	15	6.7	6.7	6.7	6.7	0	0	6.7	6.7	6.7	13.3
Siaya	16	18.8	12.5	25.0	12.5	0	0	6.3	0	0	18.8
Usigu	4	0	0	0	0	0	0	0	0	0	0
Anova (*p*)		0.877	0.954	**0.011**	0.710	-	0.290	0.799	0.681	0.595	0.404
Total	96	13.5	11.5	27.1	8.3	0	4.2	3.1	2.1	6.3	24

Df = 5 (ANOVA test between villages excluding Usigu where the count was 4); *p* value in bold indicates levels where parasite prevalence was different between villages

Table 3.3 shows the prevalence of parasites in children in the seven villages. A total of 96 children were examined in this survey. Turn up for the survey was lowest in Usigu village where only 4 children were examined. This village was subsequently omitted in the village-by-village analysis of parasite prevalence. Significant differences in parasite prevalence between villages were apparent only in *E. coli* infection (p=0.011). Infection rates were similar for all the other parasites in all villages. *E. coli* infection was lowest in Matayos (6.7%) and highest in Kombewa (61.5%) excluding Usigu. Children in Kombewa village also had high infection with hookworms (15.4%) and *A. lumbricoides* (30.8%). Only children in Nyahera and Matayos village recorded notable prevalence for both protozoa and helminths. In the other five villages, infestation was mainly limited to amoebae, *G. lamblia* and *A. lumbricoides*.

Fig 3.2 Parasite prevalence in mothers and children Exam 1

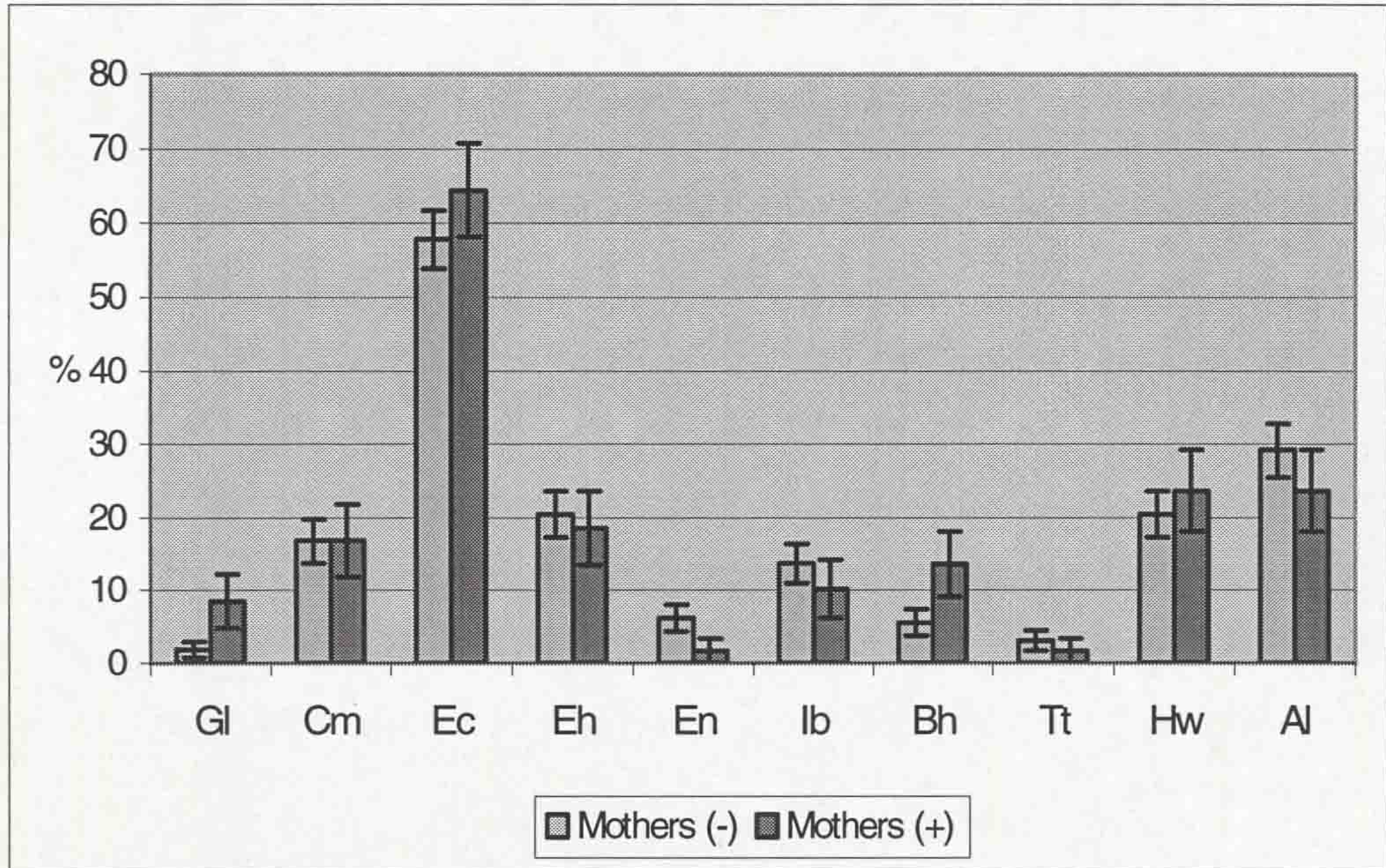


Bars represent standard error of the mean

Fig. 3.2 shows the parasite prevalence in all mothers and children sampled in Examination 1. In mothers, a prevalence of 59.4% CI ± 6.5 of *E. coli,* 20% CI ± 5.3 *E. histolytica*, and 12.7% CI ± 4.4 *I. buetschlii* were recorded. These were significantly lower in children who showed prevalence of 27%CI ± 9.1 for *E. coli*, 8.3% CI ± 5.6 for *E. histolytica* and 4.2 % CI ± 4 for *I. buetschlii*. However, children had significantly higher infections rates with *G. lamblia* (13.5% CI ± 7) compared to mothers (3.6 % CI ± 2.5). Infections with hookworms were also higher in mothers (21.4 % CI ± 5.5) compared to children (6.3% CI ± 4.9). Infection levels were similar in mothers and children for *C. mesnili, B. hominis*, *A. lumbricoides* and *T. trichiura*.

Prevalence of *C. mesnili* was unusually high in both mothers and children at 16.8 % and 11.5%. No cases of *E. nana* were detected in children. Mean parasite species count in mothers was 1.8 ± 1.4 Standard Deviation (SD), ranging from 0-6. The mean count in children was 1.1 (SD ± 1.2). Parasite prevalence was thereafter analysed in mothers and children by their respective HIV status.

Fig 3.3 Parasite prevalence in HIV-infected and HIV-uninfected mothers Exam 1

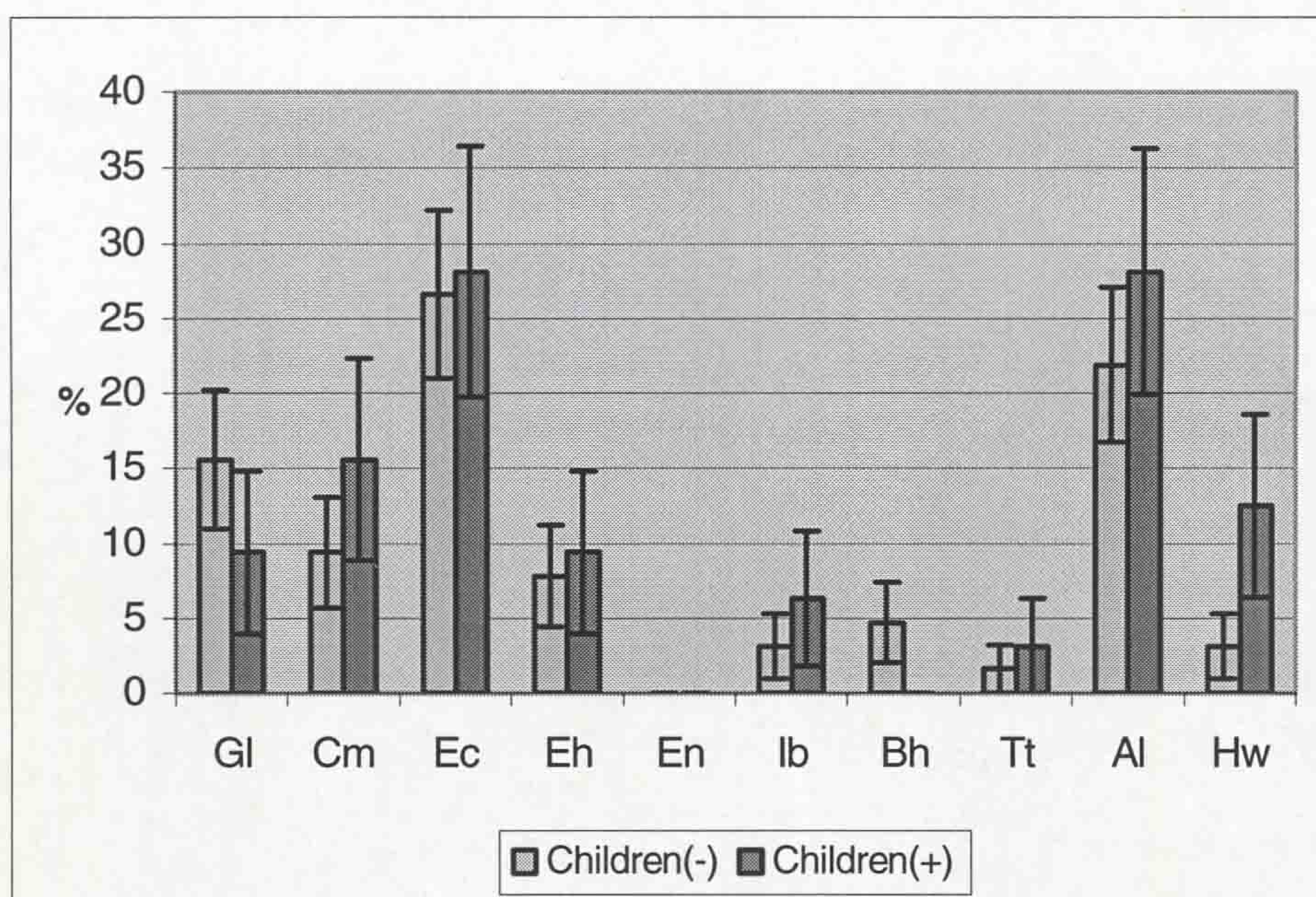


Bars represent standard error of the mean

Fig 3.3 shows the prevalence of parasites in both HIV-negative and HIV-positive mothers in Examination 1. A total of 161 (73.2%) HIV-negative and 59 (26.8%) HIV-positive mothers were examined. *Giardia lamblia* infection was significantly higher in HIV-infected mothers compared to uninfected ones (p=0.034). HIV-infected mothers also had marginally higher infection levels of *B. hominis* (p=0.05). The two groups had similar infection rates for all the other protozoa and helminths.

Mean parasite species count in HIV-infected mothers was 1.8 (SD ±1.2) ranging from 0-5 while in HIV-uninfected mothers it was 1.5 (SD ± 1.4) ranging from 0-6.

Fig 3.4 Parasite prevalence in children by HIV status Exam 1



Bars represent standard error of the mean

Fig 3.4 shows the parasite prevalence in children with or without HIV infection. A total of 64 (66.7%) HIV-negative children, and 32 (33.3%) HIV-positive children were surveyed. HIV-infected children recorded slightly higher infection rates for most parasites. A higher trend to hookworm infection was observed in HIV-infected children compared to HIV-uninfected ones (12.9 ± 12.5% vs 3.1 ± 4.4%; *p*=0.093 respectively). While more HIV-uninfected children were infected with *G. lamblia* at 15.6% compared to HIV-infected ones with a prevalence of 9.4%, the difference was small and not statistically significant.

Overall parasite species counts in HIV-uninfected children was 1.0 (SD ± 1.1) ranging from 0-5 while in HIV-infected children, it was 1.16 (SD ± 1.18) ranging from 0-3 parasite species.

Table 3.4a) Parasite prevalence in HIV-uninfected mothers and children Exam 1

		% Prevalence in HIV uninfected mothers and children									
HIV –ve	(n)	Gl	Cm	Ec	Eh	En	Ib	Bh	Tt	Hw	Al
Mothers -	161	1.9	16.8	57.8	20.5	6.2	13.7	5.6	3.1	20.5	29.2
Children -	64	15.6	9.4	26.6	7.8	0	3.1	4.7	1.6	3.1	21.9
p-value		**0.001**	0.112	**0.001**	**0.014**	**0.032**	**0.013**	0.540	0.452	**0.001**	0.172

p= values in bold show where parasite prevalence was significantly different between mothers and children (Fishers exact test)

Table 3.4 (a) shows the parasite prevalence in HIV-uninfected mothers compared to HIV-uninfected children. Significantly higher infection levels with amoebae and hookworms were observed in HIV-uninfected mothers compared to HIV-uninfected children. *G. lamblia* infection was higher among children than in mothers. Prevalence was similar for *C. mesnili, B. hominis T. trichiura* and *A. lumbricoides* in both groups.

Table 3.4b) Parasite prevalence in HIV-infected mothers and children Exam 1

		% Prevalence in HIV-infected mothers and children									
HIV +ve	n	Gl	Cm	Ec	Eh	En	Ib	Bh	Tt	Hw	Al
Mothers +	59	8.5	16.9	64.4	18.6	1.7	10.2	13.6	1.7	23.7	23.7
Children +	32	9.4	15.6	28.1	9.4	0	6.3	0	3.1	12.5	28.1
p-value		0.292	0.231	**0.001**	0.130	0.648	0.263	**0.026**	0.461	0.101	0.177

p= values in bold show where parasite prevalence was significantly different between mothers and children (Fishers exact test)

Table 3.4 (b) shows a similar comparison between HIV-infected mothers and HIV-infected children. In this category, parasite prevalence was different only for *E. coli* (p=0.001) and *B. hominis* (p=0.026). Prevalence was similar in all the other parasites in both mothers and children as shown in Table 3.4 (b).

3.4 Parasite Prevalence in Examination 2

3.4.1 Results Examination 2

A total of 249 mothers and children were able to give a stool sample in the second survey. This comprised of 168 (67.5%) mothers of whom 127 (75.6%) were HIV-negative and 41 (24.4%) were HIV-positive. Eighty-one (32.5% of total participants) children were examined in the second survey of whom 64 (79%) were HIV-negative and 17 (20.9%) were HIV-positive.

Stool examination revealed a similar occurrence of parasites as in Exam 1 and the subsequent analysis was on the ten most common parasites. Cross-sectional survey on prevalence in mothers by village was assessed as shown in Table 3.5 below.

Table 3.5. Parasite prevalence in mothers by village Exam 2

		% prevalence for each parasite									
Village	(n)	Gl	Cm	Ec	Eh	En	Ib	Bh	Tt	Hw	Al
Chulaimbo	11	9.1	18.2	72.7	27.3	5.6	18.2	0	9.1	45.5	18.2
Kombewa	34	5.9	26.5	55.9	23.5	14.7	29.4	11.8	0	8.8	11.8
Khunyangu	37	8.1	35.1	78.4	35.1	29.7	18.9	8.1	8.1	35.1	37.8
Matayos	32	12.5	34.4	75.0	40.6	34.4	18.8	15.6	3.1	40.6	43.8
Nyahera	17	0	11.8	82.4	11.8	5.9	0	0	5.9	35.3	5.9
Siaya	14	14.3	7.1	71.4	7.1	28.6	0	28.6	7.1	35.7	7.1
Usigu	23	4.3	30.4	43.5	21.7	8.7	8.7	4.3	13.0	17.4	21.7
Total	168	7.7	26.8	67.9	26.8	21.4	16.1	10.1	5.9	29.2	24.4
p value		0.697	0.274	**0.044**	0.141	0.096	0.067	0.103	0.517	**0.042**	**0.005**

Df = 6, (*p* values in bold indicates parasite prevalence levels that were significantly different between villages. ANOVA, $p > 0.05$)

Table 3.5 shows the parasite prevalence in mothers in Exam 2 within each village. Significant differences were observed between villages with Chulaimbo, Matayos, and Khunyangu recording high levels of *E. coli*, hookworms and *A. lumbricoides* compared to the other villages. Except for Nyahera, all villages recorded higher infection rates with most parasites compared to Exam 1. In Nyahera, a significant drop of *A. lumbricoides* was observed 20.9% (Table 3.2) to 5.9 % in this examination. However, there were fewer participants in this survey and therefore a lower sample size. Parasite prevalence in children was analysed by villages as shown in Table 3.6 below.

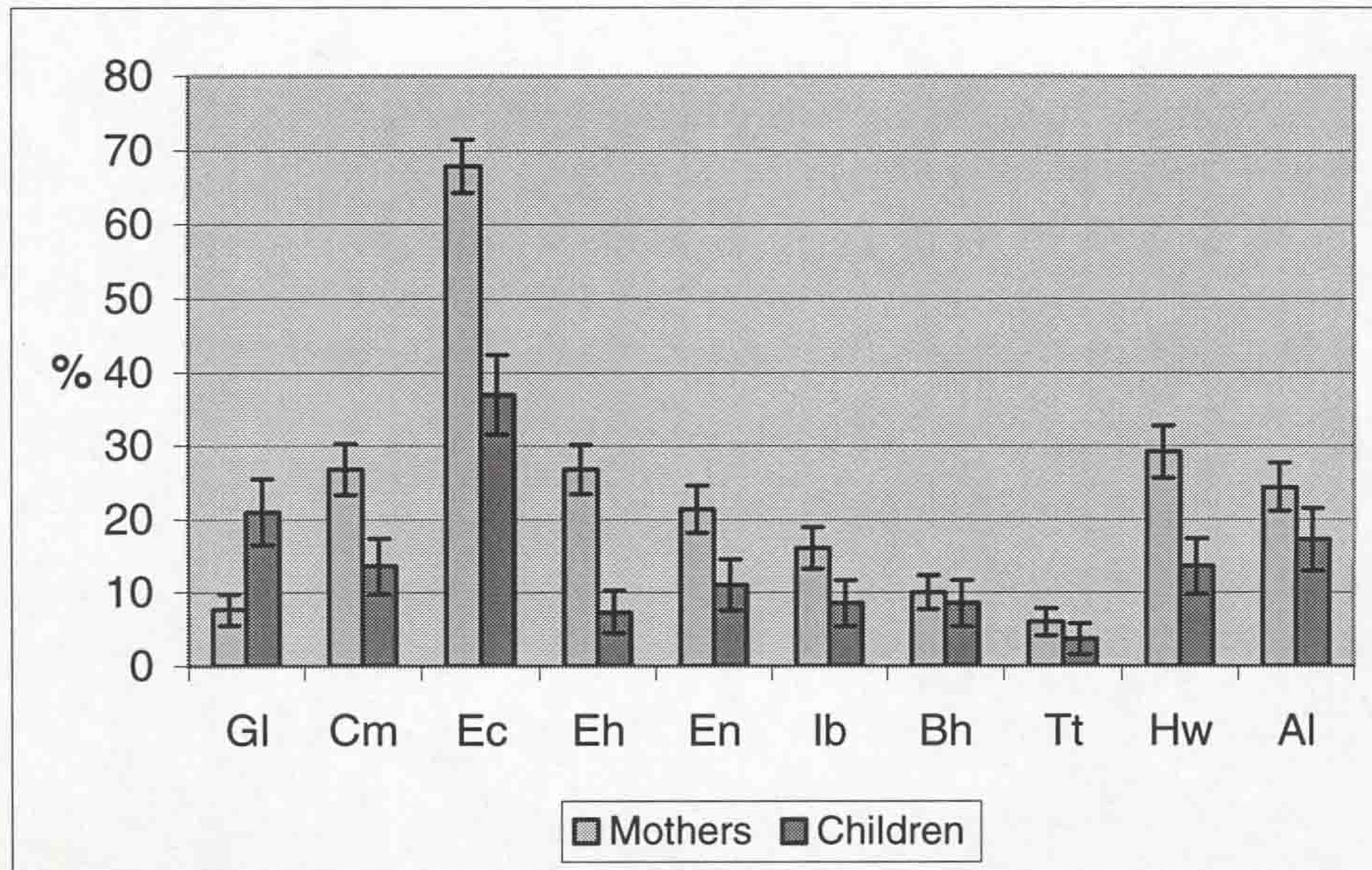
Table 3.6 Parasite prevalence in children by village, Exam 2

		% parasite prevalence									
Village	n	Gl	Cm	Ec	Eh	En	Ib	Bh	Tt	Hw	Al
Chulaimbo	5	20.0	20.0	80.0	20.0	0	40.0	0	0	0	0
Kombewa	20	25.0	10.0	35.0	5.0	5.0	10.0	5.0	0	10.0	15.0
Nyahera	21	19.0	9.5	19.0	4.8	9.5	0	4.8	9.5	9.5	14.3
Khunyangu	16	18.8	18.8	31.3	6.3	18.8	12.5	18.8	0	12.5	12.5
Matayos	4	25.0	0	75.0	0	0	0	0	0	25.0	25.0
Siaya	11	9.1	18.2	54.5	18.2	27.3	9.1	9.1	9.1	27.3	36.4
Usigu	4	50.0	25.0	25.0	0	0	0	25.0	0	25.0	25.0
Total	81	21.0	13.6	37.0	7.4	11.1	8.6	8.6	3.7	13.6	17.3
p value		0.770	0.888	0.079	0.667	0.392	0.156	0.542	0.573	0.682	0.591

Df = 6, (ANOVA all *p* values were greater than 0.05)

Table 3.6 shows the parasite prevalence in children within each village in Exam 2. In this examination, parasite prevalence in children was similar in all the seven villages surveyed. Participation was low resulting to an apparent high infection rates with *E. coli* in Chulaimbo and Matayos (80% and 75%) where five or less participants were surveyed. Prevalence of *G. lamblia, A. lumbricoides* and hookworms remained high in most villages being on average 21%, 17.3% and 13.6% respectively.

Fig 3.5 Parasite prevalence in mothers and children Exam 2

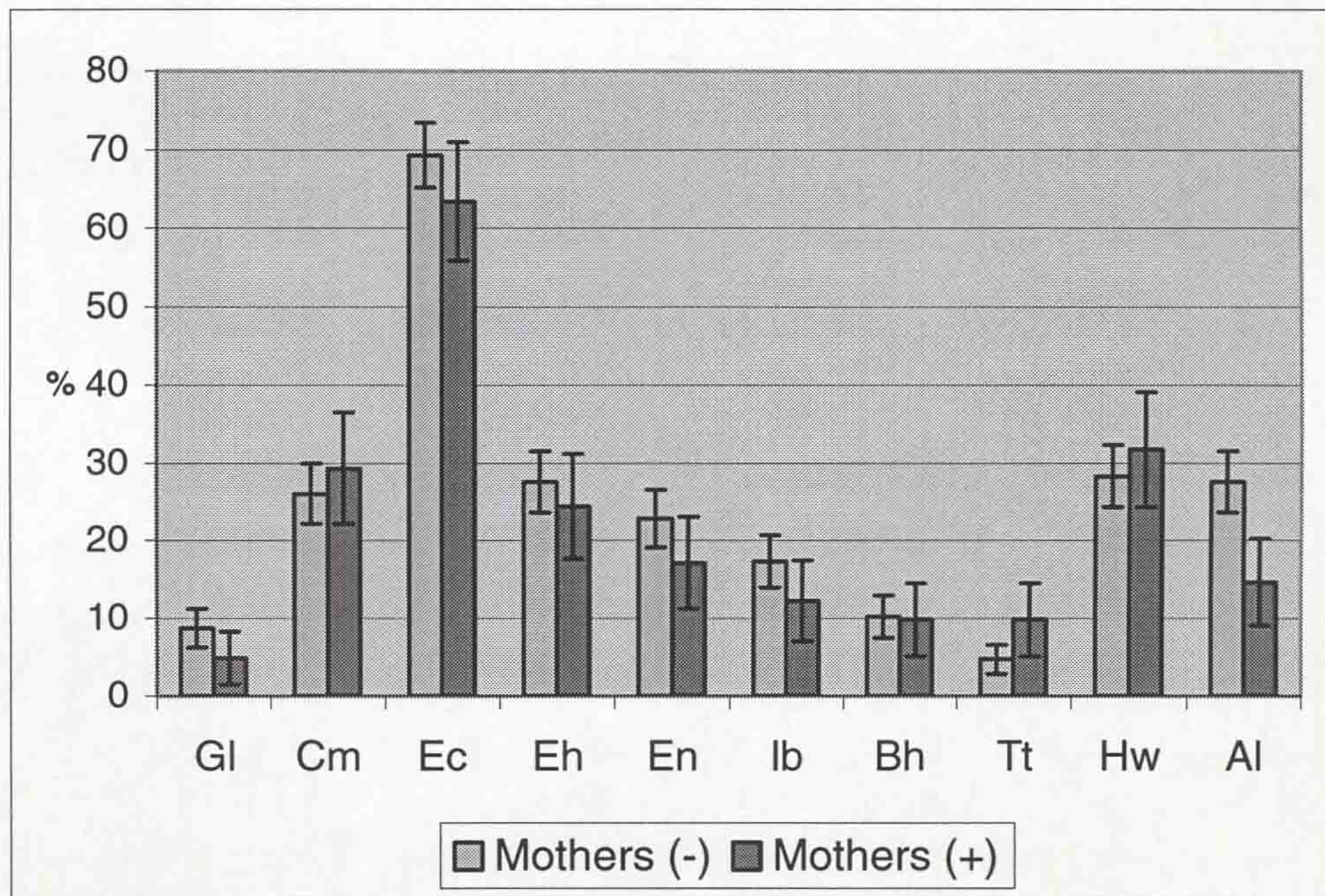


Bars represent standard error of the mean

Fig. 3.5 shows the parasite prevalence in mothers and children in Exam 2. As in Exam 1, significantly higher infection rates were recorded in mothers for *E. coli* (67.9 % CI ± 7.1), *C. mesnili* (26.8% CI ± 6.8) and hookworms (29.2% CI ± 6.9). Infection with *G. lamblia* was lower in mothers (7.7% CI ± 4.1) compared to children (21% CI ± 7.6; *p*= 0.003). Mothers and children showed similar infection rates for *I. buetschlii, B. hominis, T. trichiura*, and *A. lumbricoides*.

Overall mean parasite species counts in mothers was 2.3 (SD ±1.7) ranging from 0-6. The mean count in children was 1.4 (SD ± 1.35) ranging from 0-6 different species.

Fig 3.6 Parasite prevalence in mothers by HIV status in Exam 2

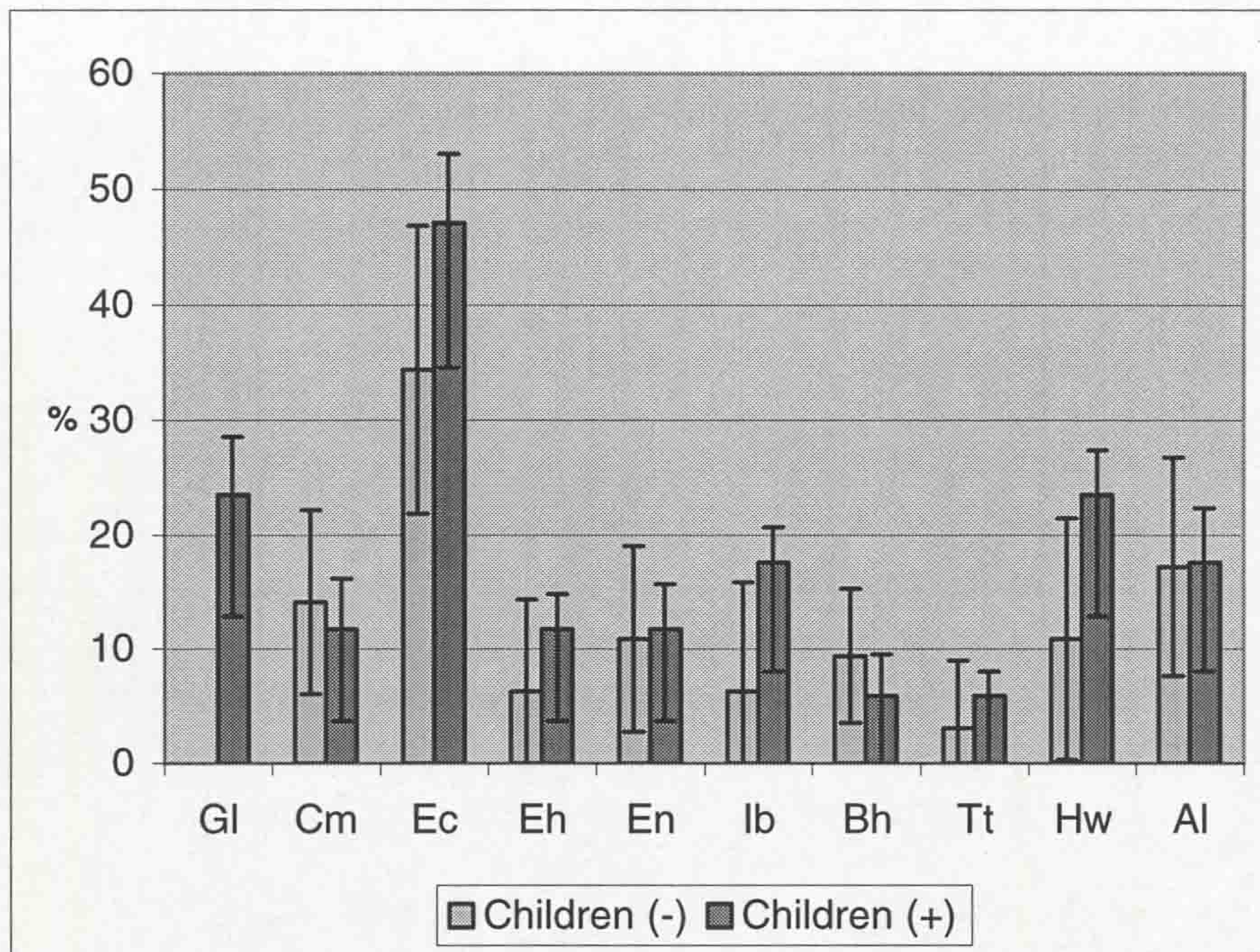


Bars represent standard error of the mean

Fig 3.6 shows the parasite prevalence in HIV-positive and HIV-negative mothers in Exam 2. Overall, there was no significant difference between HIV-positive and HIV-negative mothers. Infection rates were highest for *E. coli* at over 60% in both HIV-infected and uninfected mothers. *Chilomastix mesnili* and *E. histolytica* had prevalence of over 25% in both HIV-infected and un-infected mothers. Prevalence was lowest for *G. lamblia*, and *T. trichiura* in both groups where less than 10% prevalence was recorded.

Mean parasite species count in HIV-uninfected mothers was 2.5 (SD ± 1.7) ranging from 0-6 while the mean count in HIV-infected mothers was 2.2 (SD ± 1.6) ranging from 0-5.

Fig 3.7 Parasite prevalence in children by HIV status Exam 2



Bars represent standard error of the mean

Fig 3.7 shows the parasite prevalence in children by their HIV status in Exam 2. There were no significant differences in parasite prevalence between HIV-infected and HIV-uninfected children for all the parasites. The higher tendency of higher infection rates with hookworms observed in HIV-positive children compared to HIV-uninfected ones was again apparent in this examination at 23.5% CI ± 22.5 compared 10.9% CI ± 7.9 but the difference was not significant (p=0.169).

Mean parasite species count in HIV-uninfected children was 1.3 (SD ± 1.4) ranging from 0-6 while the mean count in HIV-infected children was 1.8 (SD ± 1.3) ranging from 0-5.

Table 3.7a) Parasite prevalence within HIV status in mothers and children Exam 2

		% parasite prevalence									
M/Ch status	N	Gl	Cm	Ec	Eh	En	Ib	Bh	Tt	Hw	Al
Mothers (-)	127	8.7	26.0	69.3	27.6	22.8	17.3	10.2	4.7	28.3	27.6
Children (-)	64	20.3	14.1	34.4	6.3	10.9	6.3	9.4	3.1	10.9	17.2
p value		**0.022**	**0.043**	**0.001**	**0.001**	**0.034**	**0.026**	0.536	0.462	**0.004**	0.078

p values in bold indicate levels of significance difference

Tables 3.7(a) and (b) shows the differences in parasite prevalence between the HIV-negative mothers and children (a) in Exam 2. As observed in Exam 1, significant difference in parasite prevalence was observed between HIV-negative mothers and HIV-negative children in almost all protozoa except *B. hominis*. Infection rates were also similar for *A. lumbricoides* and *T. trichiura.*

Table 3.7a) Parasite prevalence within HIV status in mothers and children Exam 2

M/Ch status	N	Gl	Cm	Ec	Eh	En	Ib	Bh	Tt	Hw	Al
Mothers (+)	41	4.9	29.3	63.4	24.4	17.1	12.2	9.8	9.8	31.7	14.6
Children (+)	17	23.5	11.8	47.1	11.8	11.8	17.6	5.9	5.9	23.5	17.6
p value		0.06	0.14	0.20	0.24	0.47	0.43	0.54	0.54	0.39	0.53

Degrees of freedom =1,
p= values in bold show where parasite prevalence was significantly different between mothers and children

Table 3.7 b) shows the parasite prevalence in HIV-positive mothers and children in Exam 2. Mothers and children had similar infection rates for all parasites. There was a higher trend for *G. lamblia* infection in children (23.5%) compared to mothers (4.9%; *p*=0.055).

3.5 Parasite Prevalence in Examination 3

3.5.1 Results Examination 3

The third survey had the lowest turn up with only 193 participants coming forth for examination. They included 116 (60.3%) mothers and 77 (39.7%) children. A total of 87 (75%) mothers were HIV-negative and 29 (25%) were HIV-positive while among the children, 68 (88.3%) were HIV-negative and 9 (11.6%) were HIV-positive. Only 1 participant turned up for examination in Chulaimbo while 2 turned up in Usigu villages. The two villages were therefore omitted in the village-by-village analysis. Parasite prevalence was analysed among mothers and children by village and by HIV status as in the earlier examinations

Table 3.8 Prevalence of parasites in mothers by village Exam 3

		% prevalence for each parasite									
Village	(n)	Gl	Cm	Ec	Eh	En	Ib	Bh	Tt	Hw	Al
Kombewa	17	0	5.9	70.6	23.5	5.9	5.9	5.9	0	17.6	23.5
Khunyangu	24	12.5	29.2	62.5	29.2	12.5	4.2	12.5	0	25.0	29.2
Matayos	17	11.8	29.4	52.9	35.3	17.6	17.6	17.6	11.8	23.5	35.3
Nyahera	15	20.0	13.3	80.0	33.3	13.3	13.3	20.0	0	33.3	6.7
Siaya	40	0	10.0	60.0	32.5	10.0	10.0	30.0	2.5	12.5	12.5
Total	113	7.1	16.8	63.7	31.0	11.5	9.7	19.5	2.7	20.4	20.4
p value		**0.044**	0.114	0.529	0.950	0.858	0.632	0.230	0.136	0.465	0.144

Df = 4, (ANOVA $p>0.05$) *p* values in bold show significantly different parasite prevalence levels between villages

Table 3.8 shows the parasite prevalence in mothers for each of the five villages analysed in the third survey. A marginal difference in prevalence was observed for *G. lamblia* in mothers between the five villages (p=0.044). Prevalence was highest in Nyahera village at 20%, the highest *G. lamblia* infection recorded in mothers throughout the examinations. In

the third survey parasite prevalence among children remained unchanged in most villages as shown in Table 3.9 below.

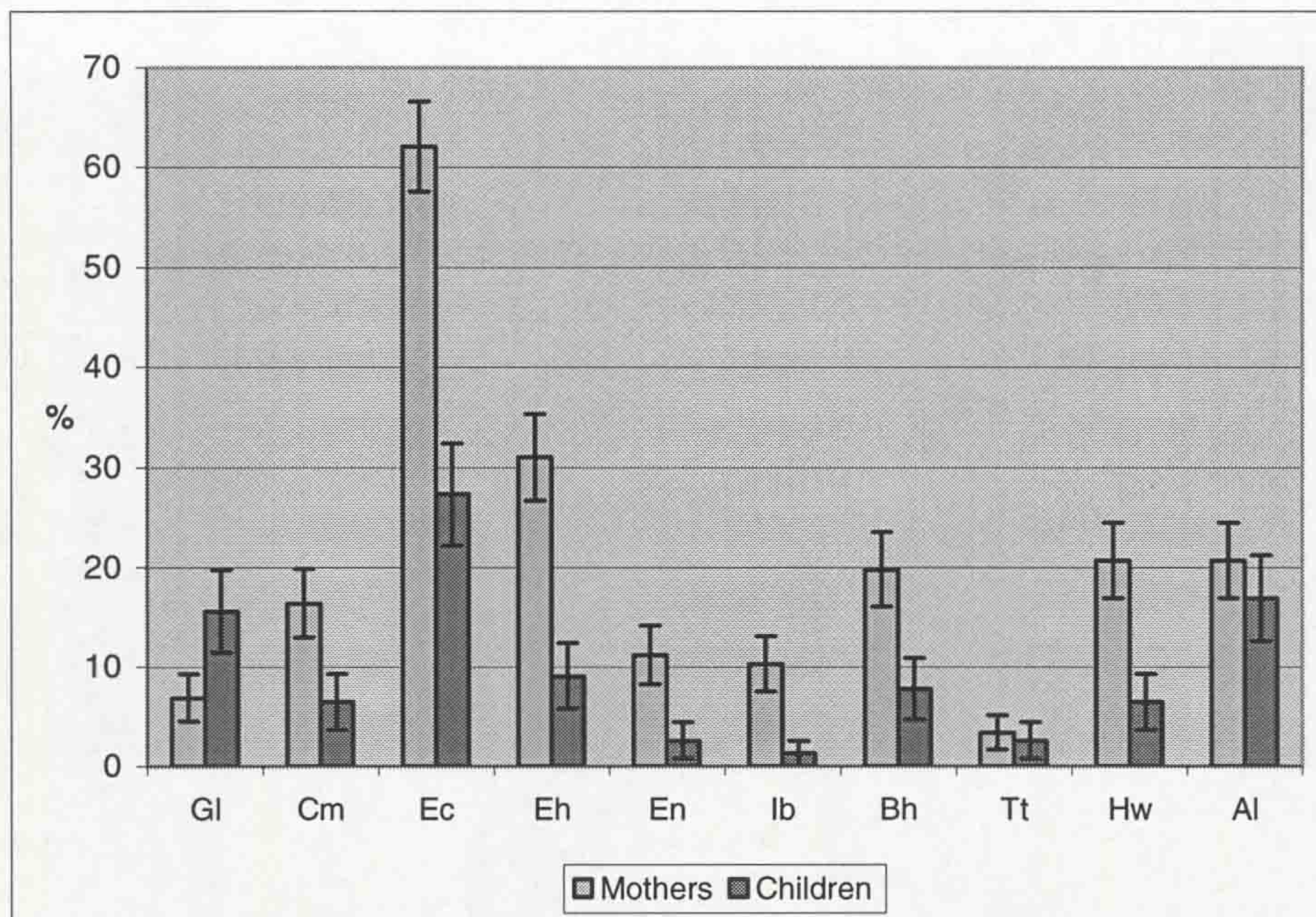
Table 3.9. Parasite prevalence in children by village Exam 3

		% prevalence for each parasite									
Village	(n)	Gl	Cm	Ec	Eh	En	Ib	Bh	Tt	Hw	Al
Kombewa	7	14.3	14.3	42.9	0	0	0	14.3	0	14.3	28.6
Khunyangu	9	11.1	0	33.3	0	0	0	0	0	0	11.1
Matayos	15	26.7	0	26.7	6.7	6.7	0	0	6.7	0	13.3
Nyahera	11	9.1	9.1	9.1	0	0	0	0	0	18.2	27.3
Siaya	33	12.1	9.1	27.3	15.2	3.0	3.0	15.2	3.0	6.1	15.2
Total	75	14.7	6.6	27.9	7.9	2.6	1.3	7.9	2.6	6.7	17.3
p value		0.18	0.72	0.36	0.44	0.89	0.93	0.32	0.84	0.43	0.83

Df= 4, (ANOVA all $p>0.05$)

Table 3.9 shows the parasite prevalence among children in the five villages analysed. Only 5/7 villages surveyed were included in the analysis due to the low number of participants from Usigu and Chulaimbo villages. No significant differences in parasite prevalence between villages were observed. The most common parasites in all the villages were *G. lamblia, E. coli* and *A. lumbricoides*. Analysis was thereafter done by HIV status of the mothers and children.

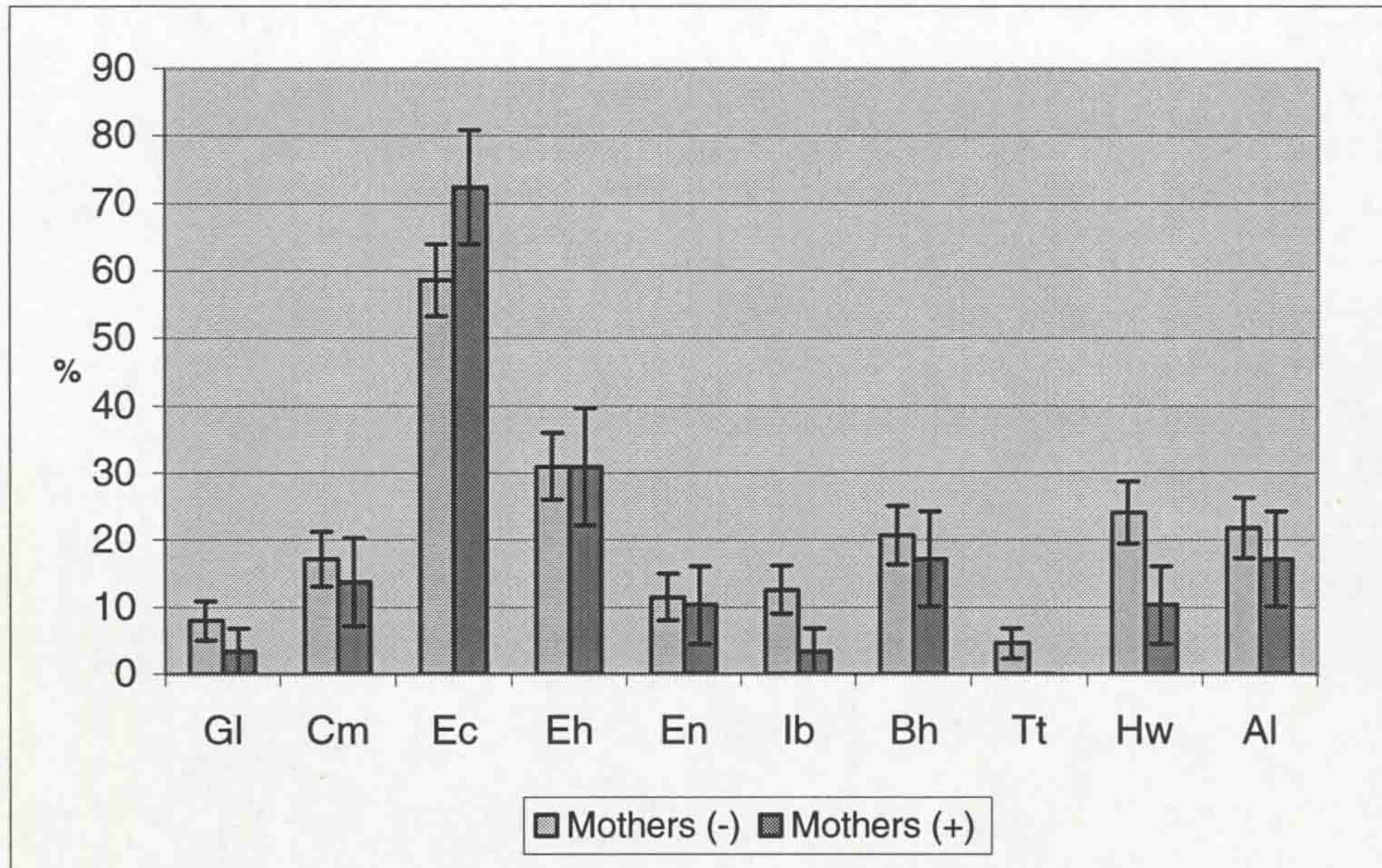
Fig 3.8 Parasite prevalence in mothers and children in Exam 3



Bars represent standard error of the mean

Fig. 3.8 shows the parasite prevalence in all the mothers and children examined in the third survey. As in the earlier examinations, highly significant differences were observed in all parasite prevalence between mothers and children except for *A. lumbricoides* (mothers 20.7%; children 16.9%; p= 0.321). Unusually high prevalence of *B. hominis* at 19.8 ± 7.4% was recorded in mothers. Again infection rates for *G. lamblia* was significantly higher in children (17.5%) than in mothers with a prevalence of 7% (p=0.034). Further parasite prevalence by HIV status was analysed in mothers and children for the third examination.

Fig 3.9 Parasite prevalence in mothers by HIV status Exam 3

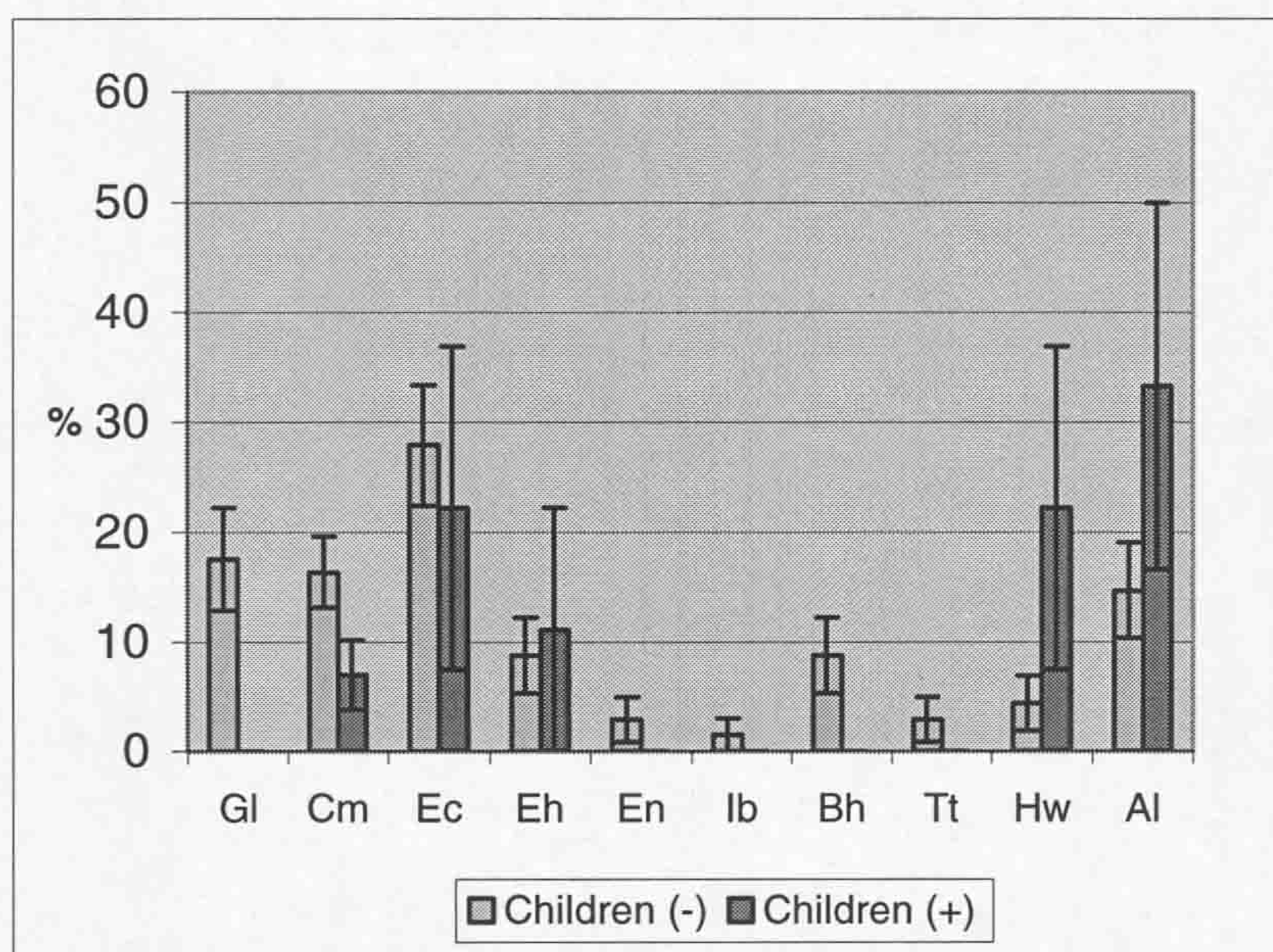


Bars represent standard error of the mean

Fig 3.9 shows the parasite prevalence in mothers in Exam 3 by their HIV status. As in Exam 1 and 2 parasites prevalence was similar in mothers with or without HIV-infection. Infection rates remained high with *E. coli* at 72.4 ± 17% in HIV-infected mothers and 58.6 ± 10.6% in HIV-uninfected ones. High infection rates with *B. hominis* were observed in all mothers regardless of the HIV status. No infections with *T. trichiura* were recorded in HIV-infected mothers.

Mean parasite species count was 1.9 (SD ± 1.2) in HIV-infected mothers ranging from 0-4. The count in HIV-uninfected mothers was 2.2 (SD ± 1.7) ranging from 0-8.

Fig 3.10 Parasite prevalence in children by HIV status in Exam 3



Bars represent standard error of the mean

Fig 3.10 shows the parasite prevalence in children in the third survey by HIV status. The survey recorded the lowest number of HIV-positive children in all the three surveys (n=9). However, the overall pattern of infections was similar to that in the two previous examinations. There was no significant difference in prevalence of hookworms by HIV status although there was a tendency to higher infection rates among HIV-positive children at 22.2% compared to 4.4% in HIV-negative ones.

Mean parasite species count in this survey was 1 (SD ± 1.4) among the HIV-infected children ranging from 0-4, while it was 0.97 (SD ± 1) among the HIV-uninfected children ranging from 0-4.

Table 3.10a) Parasite prevalence within HIV status in mothers and children Exam 3

		% parasite prevalence									
HIV –ve	N	Gl	Cm	Ec	Eh	En	Ib	Bh	Tt	Hw	Al
Mothers (-)	87	8.0	17.2	58.6	31.0	11.5	12.6	20.7	4.6	24.1	21.8
Children (-)	68	17.6	7.4	27.9	8.8	2.9	1.5	8.8	2.9	4.4	14.7
p-value		0.060	0.055	**0.000**	**0.001**	**0.043**	**0.008**	**0.034**	0.463	**0.000**	0.178

Df =1, *p*= values in bold show where parasite prevalence was significantly different between mothers and children (Fishers exact test)

Tables 3.10 (a and b) show the parasite prevalence in mothers and children examined in the third and last survey, by HIV status. As observed earlier, HIV uninfected mothers had significantly higher infection rates than children for the amoebae, *B. hominis, T. trichiura* and hookworms ($p>0.05$) while infections with *A. lumbricoides* and *T. trichiura* were similar in both groups.

Table 3.10b) Parasite prevalence within HIV status in mothers and children Exam 3

HIV +ve	N	Gl	Cm	Ec	Eh	En	Ib	Bh	Tt	Hw	Al
Mothers +ve	30	3.3	16.7	73.3	30.0	10.0	3.3	16.7	0	10.0	20.0
Children+ve	9	0	0	22.2	11.1	0	0	0	0	22.2	33.3
p-value		0.769	0.248	**0.009**	0.250	0.444	0.769	0.248	-	0.324	0.338

Df = 1; *p*= values in bold show where parasite prevalence was significantly different between mothers and children (Fishers exact test)

Among the HIV-infected group (Table 3.10b), there was again no significant difference between the HIV-infected mothers and HIV-infected children except for *E. coli* where significantly higher infection rates was observed in mothers (73.3%) compared to children (22.2%; $p=0.009$).

3.6 Summary of Results of Point Prevalence

In summary, over the three examinations, protozoa and helminths were common in all the seven villages examined. *E. coli* was the most prevalent parasite in both mothers and children in all the three examinations. Mothers had significantly higher infection rates with *E. coli, E. histolytica, E. nana* and *B. hominis* than children (p>0.017). Prevalence was similar in mothers and children for *C. mesnili, A. lumbroicoides* and *T. trichiura*. Children had significantly higher prevalence with *G. lamblia* than mothers in all the three surveys (p>0.01). Prevalence of *B. hominis* was significantly higher in mothers 20% CI ± 7.4 (p>0.034) than in children in the third examination

Overall, there were no significant differences in parasite prevalence between HIV-infected and uninfected mothers. This was also observed in HIV-infected and HIV-uninfected children where both groups showed similar infection rates with all the parasites analysed. However, there was s slight tendency for HIV-infected children to have higher hookworm infection than HIV-uninfected ones especially in the first and third examination (p=0.093)

Comparing mothers and children within their respective HIV status, HIV-infected mothers and children had similar infection rates for both protozoa and helminths. This was in contrast with the HIV-uninfected mothers and children who showed significantly diverse infection rates for most parasites.

Overall, number of different parasites species per individual was higher in mothers (both HIV-infected and uninfected) with a mean count of 2.1 (SD ± 1.53) while children had a mean count of 1.13 (SD ± 1.2). The difference in mean parasite species counts was no significant.

3.7 Period Prevalence

Principle

Period prevalence was defined as the proportion of the total number of people found positive for each parasite at least once over all the people examined during each specified period. A three and six month period prevalence were analysed during the seven months study period.

3.7.1 Seasonal Effects

The examination period covered a span of 7 months that coincided with the cold and dry period of June/July, the wetter period of short rains in Sept/Oct and the hot and dry period of Jan/Feb in the final examination. Therefore, infection trends shown below both point and period prevalence coincided with these seasonal variations. However, the survey period was not long enough to cover an annual cycle for a complete season variation.

3.7.2. Materials and Methods

3.7.2.1 First 3–months period prevalence

In the first three months period prevalence, a total of 149 participants were examined during the first and the second examination of which 98 (65.6%) were mothers and 51

(34.2%) were children. Within these groups, 75 (76.5%) mothers were HIV-negative while 23 (23.5%) were HIV-positive. Among the children 34 (66.7%) of the children were HIV-negative and 17 (33.3%) were HIV-positive.

3.7.2.2 Six months period prevalence

The third period prevalence was computed for the six months that covered the duration of the field study time. This included all participants who attended the three examinations. It was computed as the proportion of all persons positive for each of the parasites at least once in all the three examinations. A total of 61 participants attended all the three examinations that included 37 (60.7%) mothers of whom 30 (81.1%) were HIV-negative and 7 (18.9%) were HIV-positive. There were 24 (39.3%) children attending all the three examinations of whom 17 (70.8%) were HIV-negative and 7 (29.1%) were HIV-positive.

3.7.3.Results of the Period Prevalence

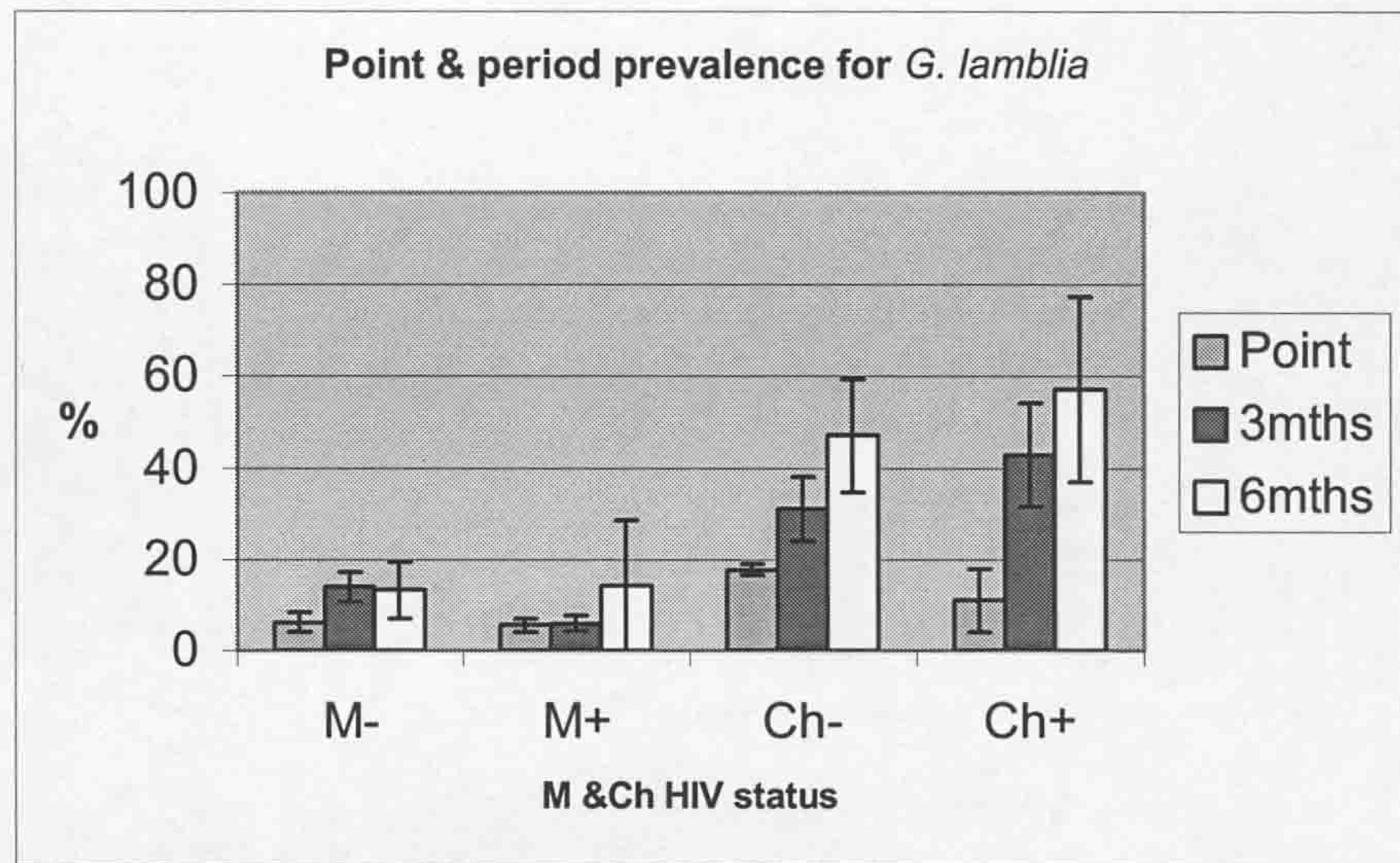
The results of the three months and six months prevalence are summarised in Figs 3.11 (a-j) below. The three months period shown are for the first three months as this period commenced at the start of the subsequent six months period assessed. While prevalence of *G. lamblia* was on average 20% among HIV-infected children it was 42.4% and 57.1% respectively in the three and six months period (Fig 3.11a). This showed that over half of the children were infected with *G. lamblia* within the six months of examination. The high increase in infection rate in children with *E. coli* was also apparent reaching over 88% in HIV-negative children (Fig3.11c). The results reveal a high incidence of *G. lamblia* and *E. coli* over the three and six months period. On the contrary, the rise in period prevalence in

E. coli was only moderate in mothers. This suggested longer period of infections than increased incidence for the six months in mothers compared to children.

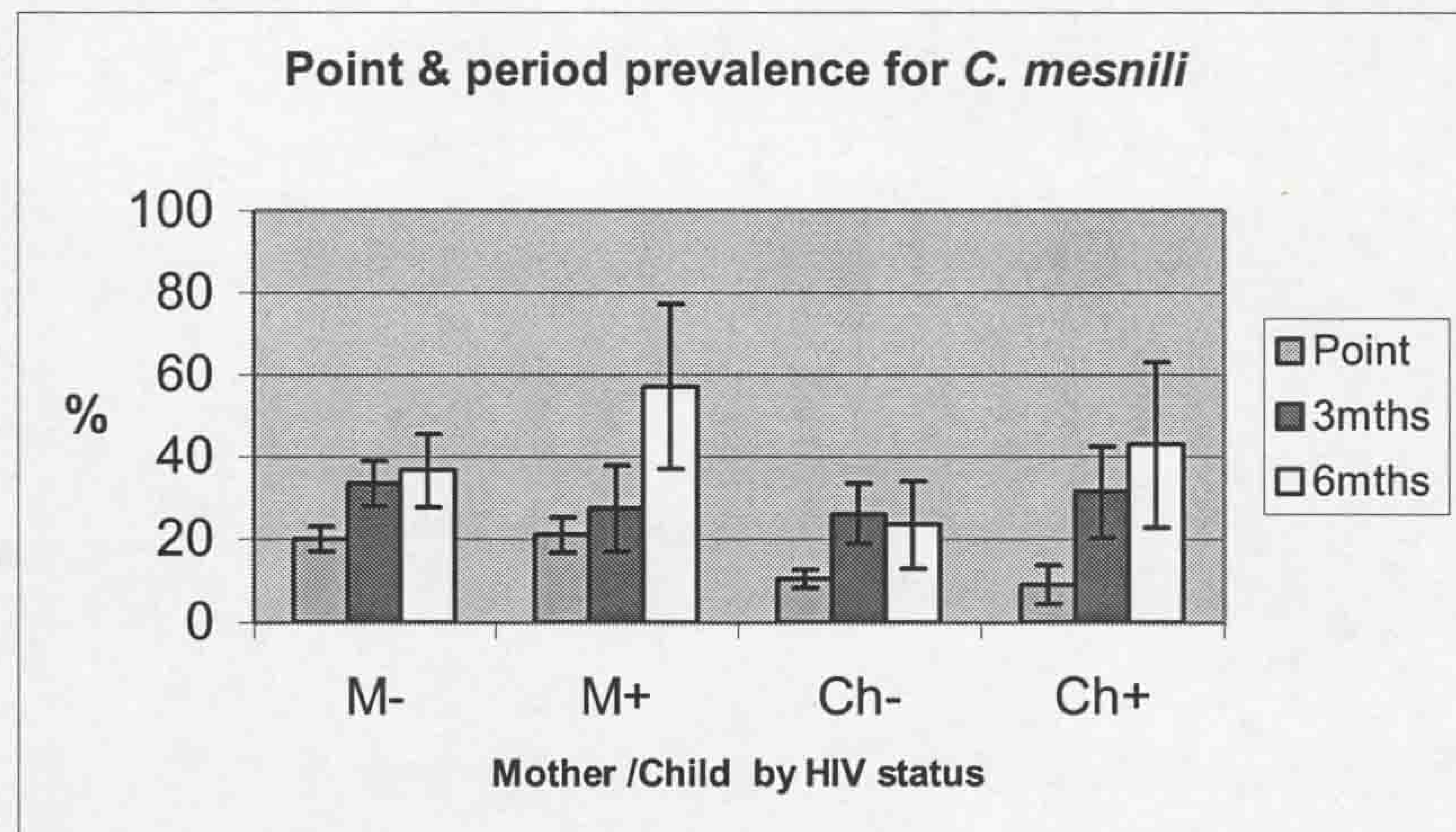
Differences in infection rates with hookworms were again apparent in the three and six months period (Fig.3.11g). In the six months period, prevalence in these children was 71.4 % while it was only 47.1% in HIV-negative ones. However, the large confidence intervals again limited the reliability of conclusive differences between the groups.

Fig 3.11 Summary of point and period prevalence in mothers and children by HIV status

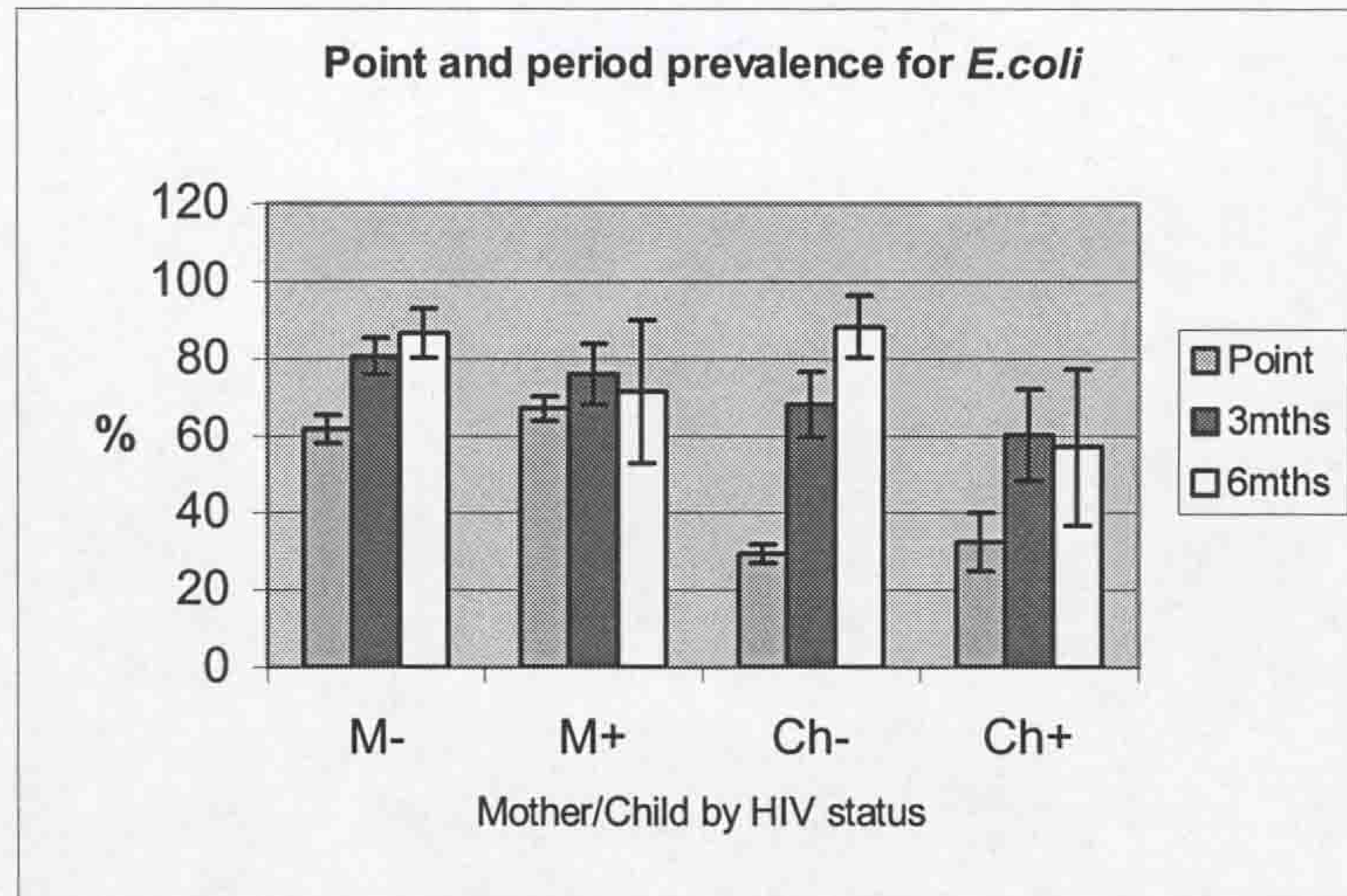
a)



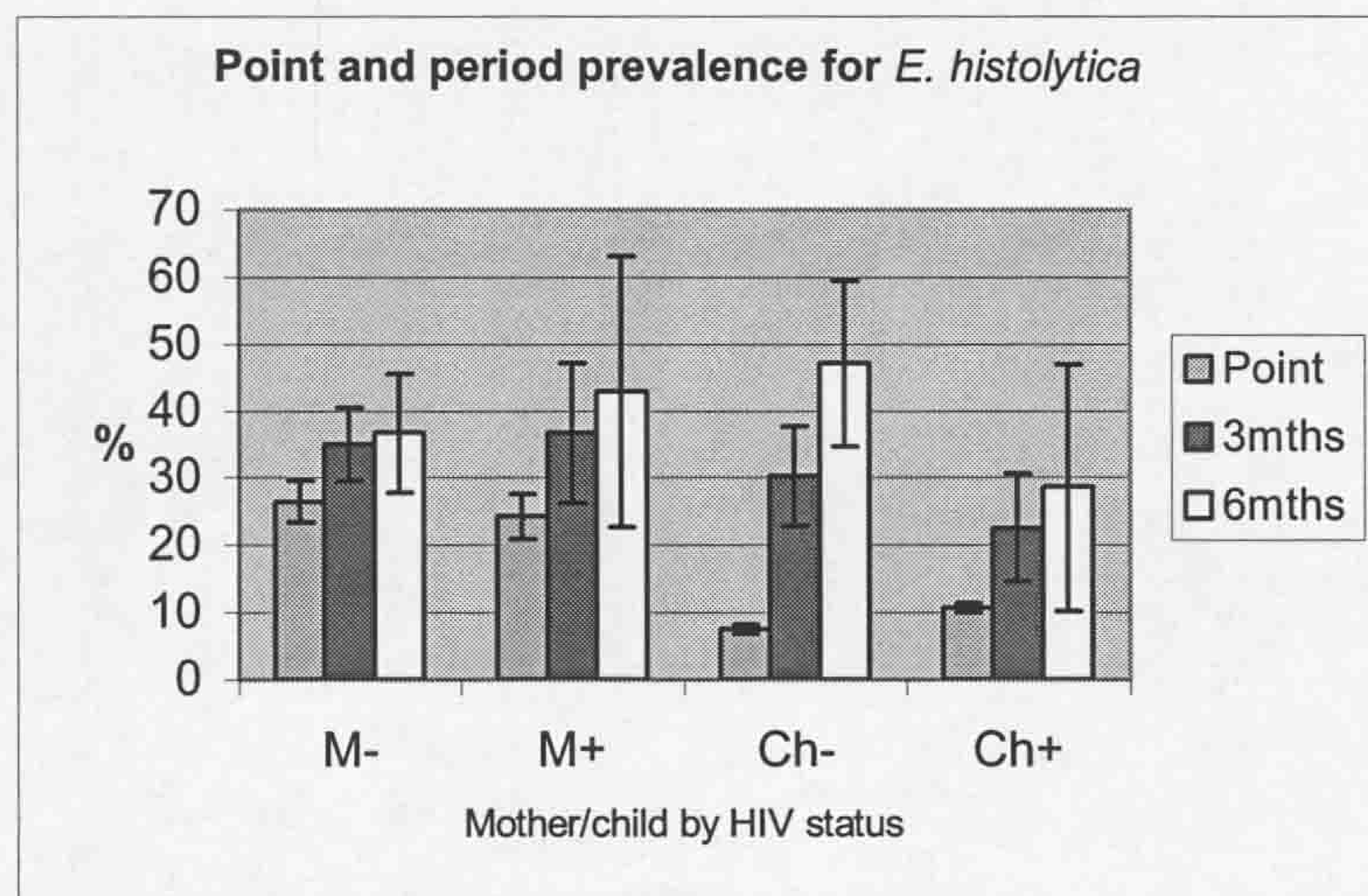
b)



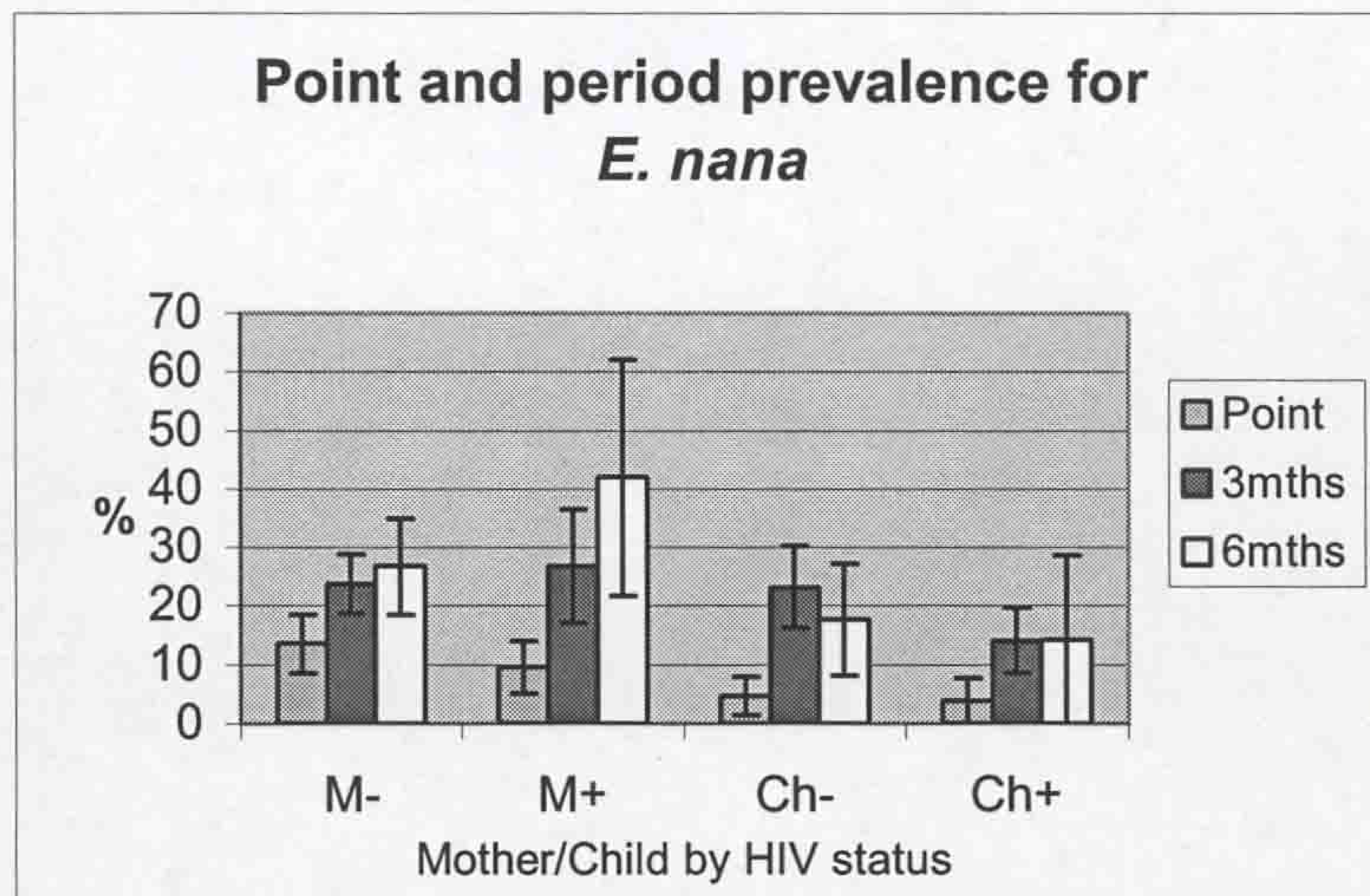
c)



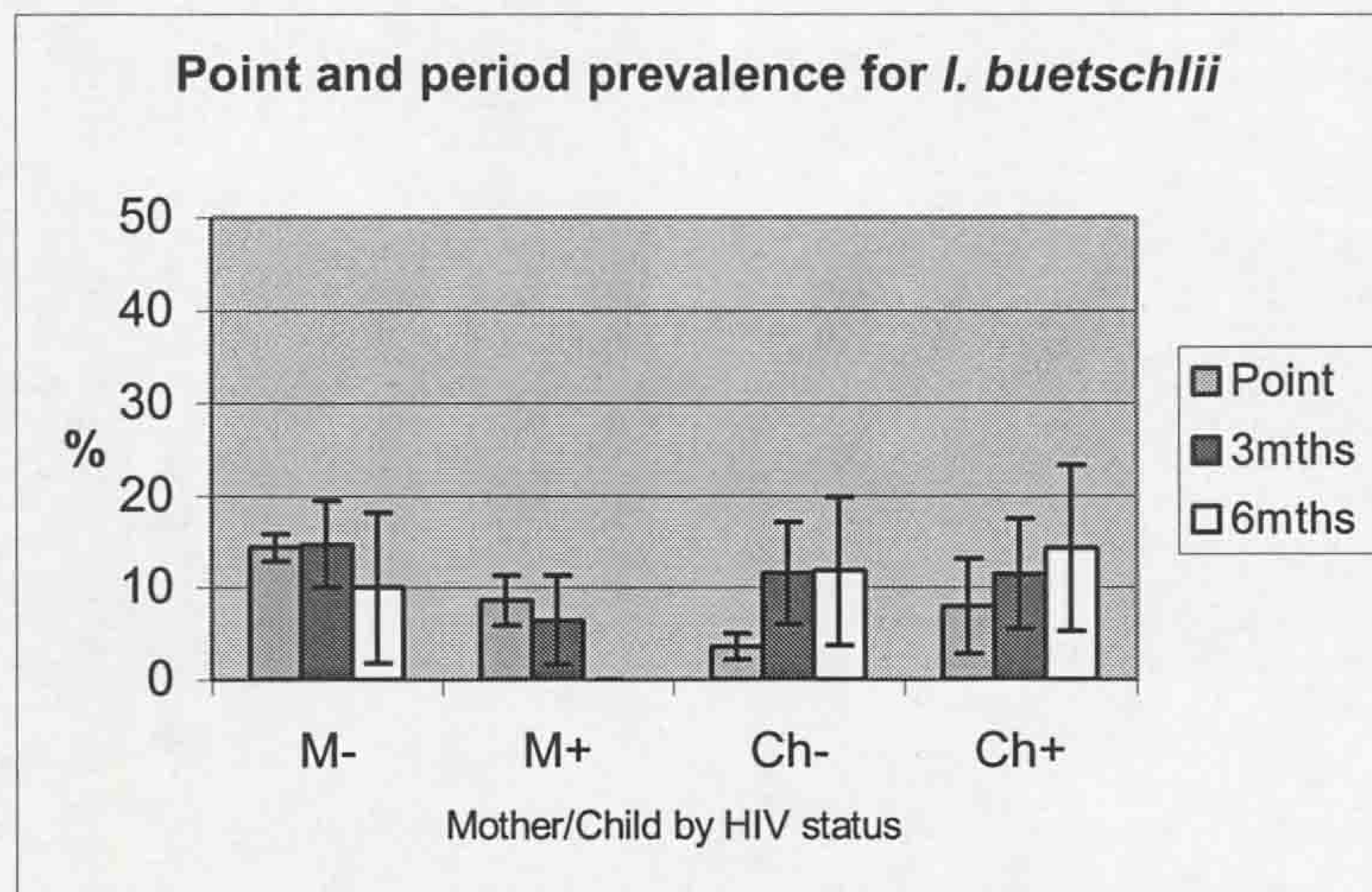
d)



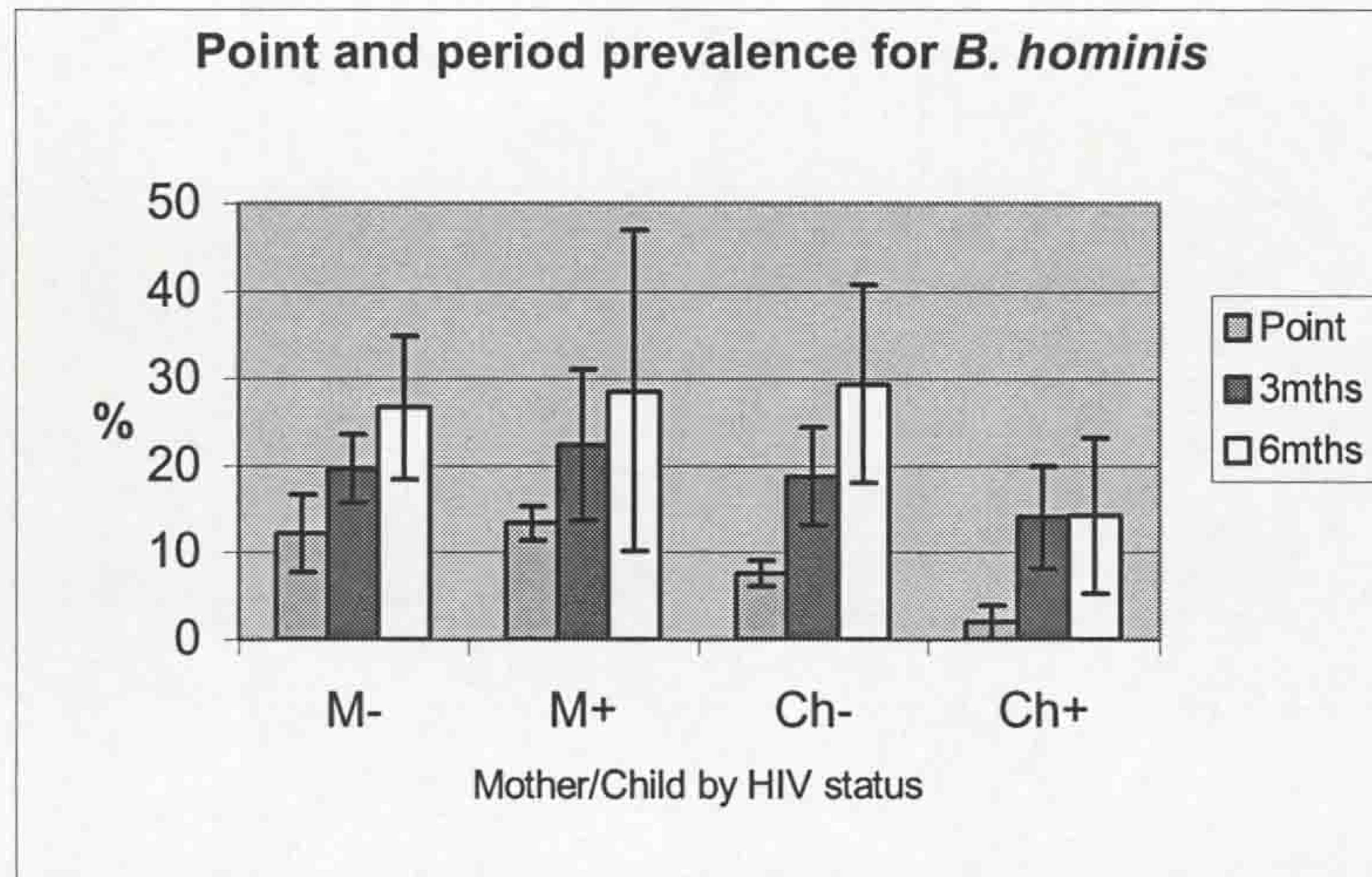
e)



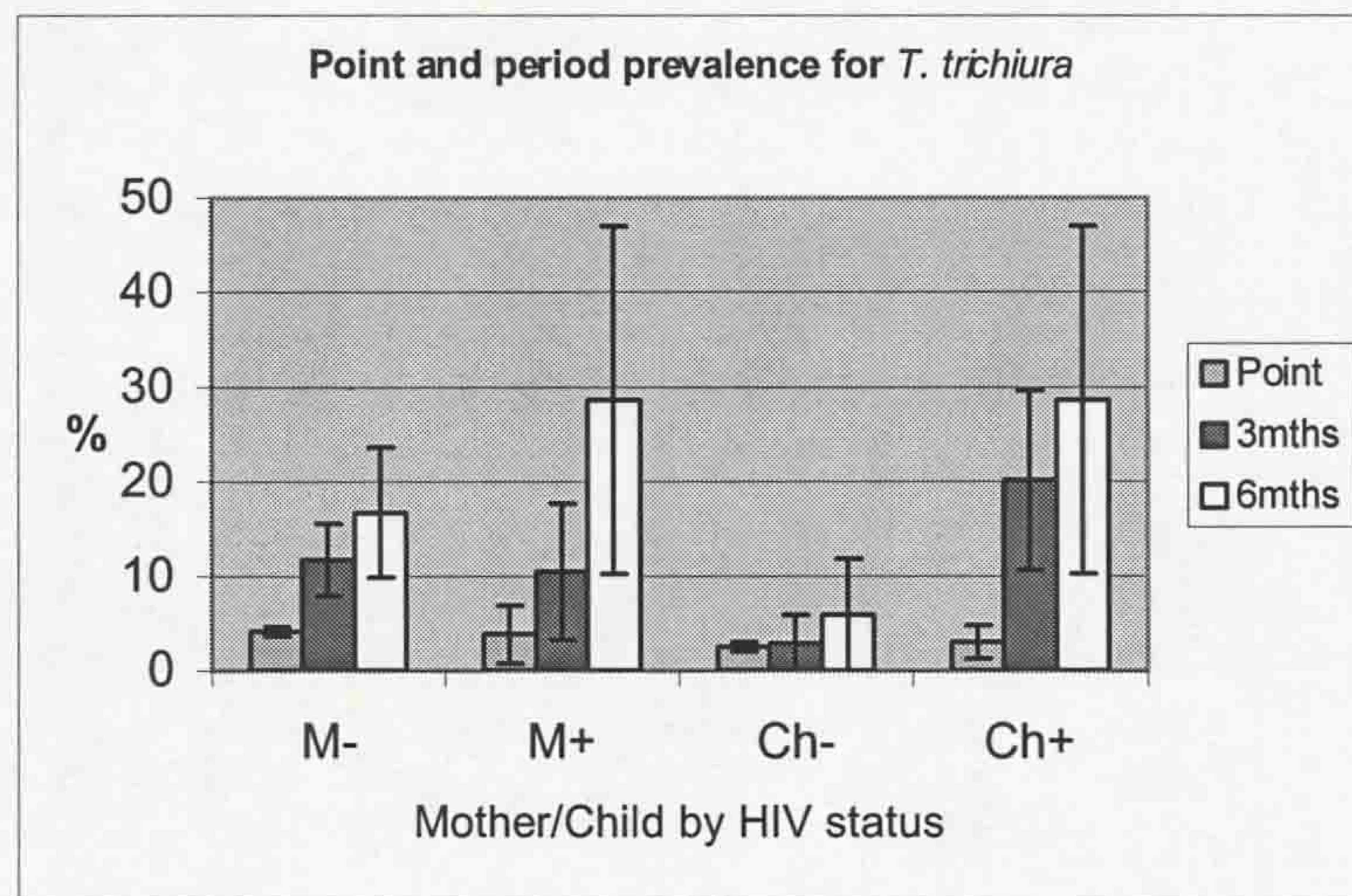
f)



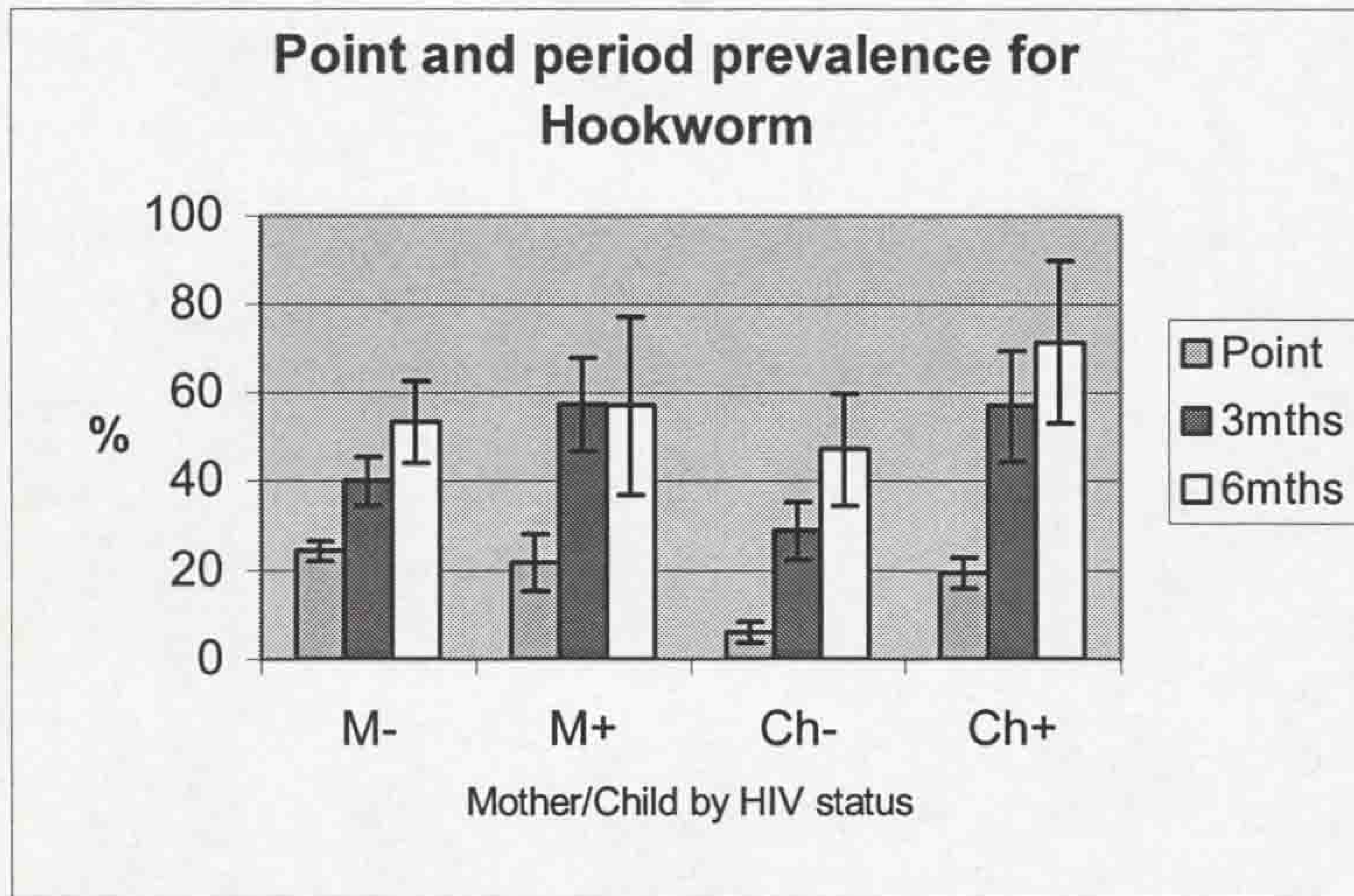
g)



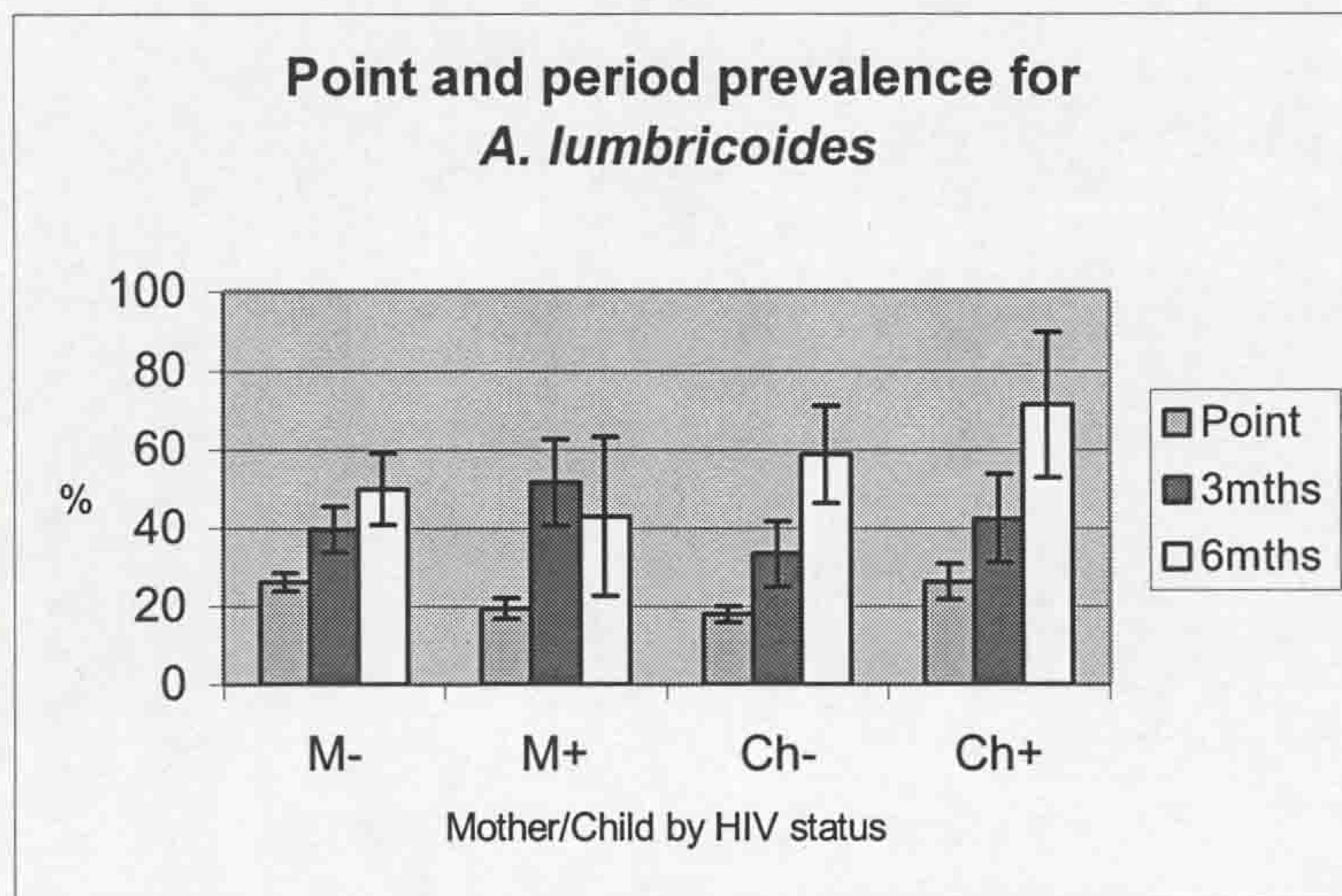
h)



i)



j)



3.8 Multiple Parasite Infections

3.8.1 Introduction

During the three cross-sectional surveys, it was apparent that the individual mothers and children harboured a wide range of different species. The frequency and proportion of these different species in sample examined was analysed in detail for the three examinations and by HIV status as shown below.

3.8.2 Results

Occurrence of multiple parasites was apparent in the three examinations. Over 77% of all the samples examined were positive with at least one parasite (Table 3.11). Different individual species identified per sample ranged from 1 to 8 counts. On average, less than 17% of the mothers recorded no parasites in the three examinations. However children were less parasitised and no parasite were recorded in 40% of the samples examined in Examination 1 and 3, while 27.2% had no parasites in Examination 2. A small proportion of the participants (5.7%) had at least 5 parasites with one person having the highest number of 8 different species in one episode.

Table 3.11. Proportion of different species per sample in mothers and children

			Sum of individual parasites per person (in %)								
Visit	M/Ch	N	0	1	2	3	4	5	6	8
1	Mother	220	16.4	30.9	29.5	11.4	6.8	3.6	1.4	0
	Children	96	44.8	26.0	15.6	11.5	1.0	1.0	0	0
2	Mother	168	13.7	20.2	21.4	18.5	13.7	8.3	4.2	0
	Children	81	27.2	33.3	21.0	12.3	1.2	3.7	1.2	0
3	Mother	116	15.5	22.4	23.3	22.4	10.3	2.6	2.6	0.9
	Children	77	40.3	33.8	18.2	3.9	3.9	0	0	0
Total	**All**	**758**	**22.8**	**27.2**	**23.0**	**14.0**	**7.3**	**3.8**	**1.8**	**0.1**

Table 3.11 shows the sum of different species identified in mothers and children in the three examinations. Most people had 1 (27%) or 2 (23%) parasites in all the examinations. Over 80% of mothers and over 60% of the children had at least one or more parasite in all the examinations. 27% of all the participants had on average three or more parasites over the six months study period.

Fig 3.12 Frequency of multiple parasites in mothers and children by HIV status

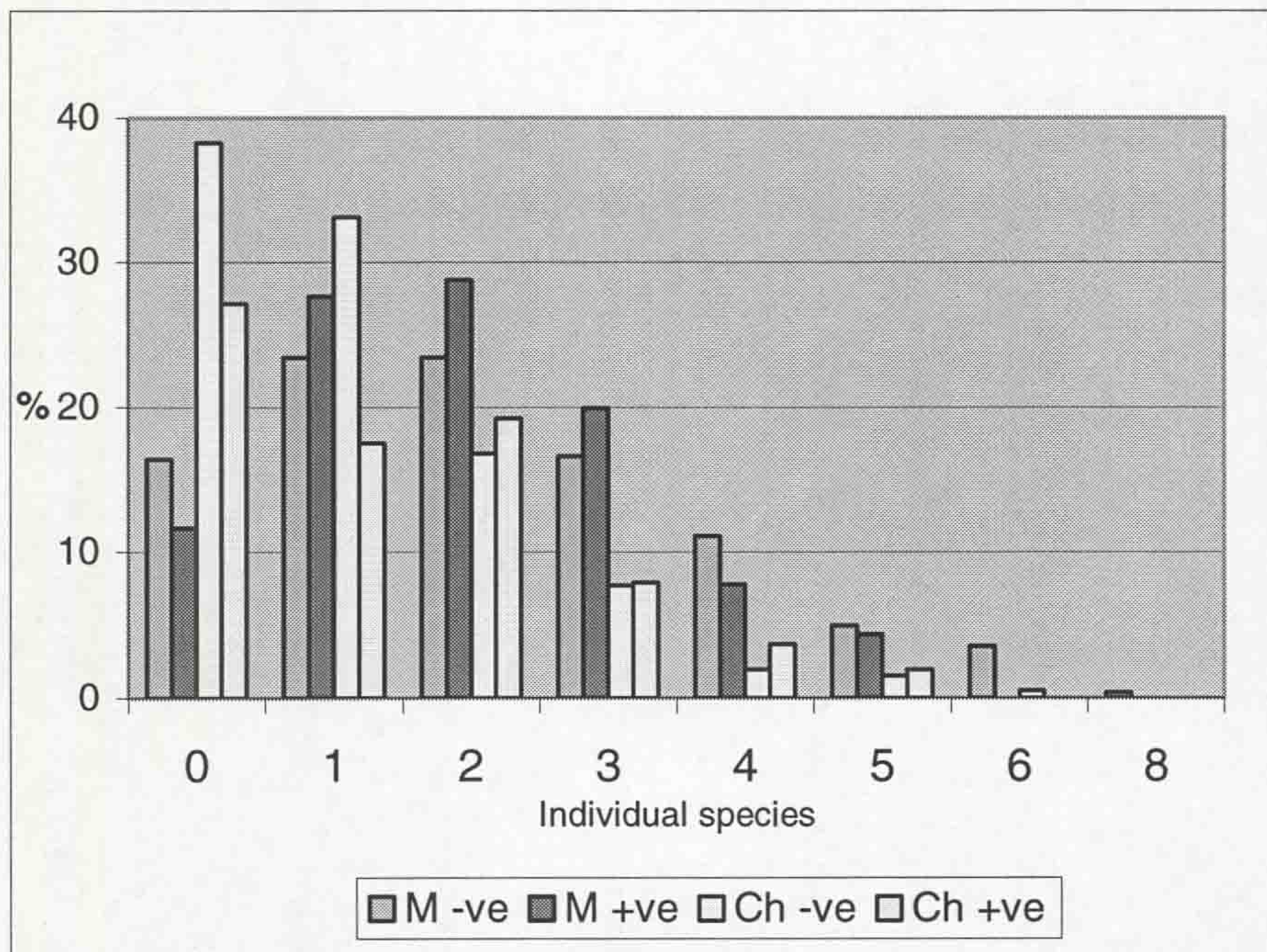


Fig 3.12 shows the frequency of individual parasite species in mothers and children by the HIV status. Mothers (both HIV-infected and uninfected) had the largest proportion of different parasite species, majority having more than one. The overall mean parasite species count in HIV-uninfected mothers was 2.5(SD ± 1.4) while in HIV-infected mothers was 2.2 (SD ± 1.3). In HIV uninfected children, it 1.9 (SD ± 1.1) while in HIV-infected ones it was 2 (SD ± 1.1). 38.3% of HIV-negative children had no parasites compared to 27.1% in HIV-infected children but the difference was not significant.

3.8.3 Summary of Results: Prevalence and Multiple Parasite Infections

So far, infection rates differed significantly only by age where mothers showed higher prevalence and higher count of individual parasite species per examination. Results on period prevalence however, suggested incidence was high in the community even for parasite that had showed low point prevalence such as *E. coli* in children or *T. trichiura*. Moreover, higher trends of hookworm infections among children were inconclusive using the Chi-square tests in both point and period prevalence. Further analyses were therefore, undertaken to investigate the overall effects of all the independent variables (village, visits, HIV) on the outcome of the infections in both mothers and children

3.9 Risk Estimates for Parasitism in the Community

Principle

Prevalence results showed no significant differences in the occurrence of most parasites in mothers or children by HIV status. There were also no differences in most parasite prevalence between villages or between the three examinations. The only significant differences observed were those between mothers and children due to their age difference. However, it was not possible to rule out the effects of HIV on infection rate, as the analysis did not include assessment for any risk factors in the variables.

Further analyses were therefore, undertaken using logistic regression. Logistic regression was used to predict the presence or absence of an infection based on the effect of visit, village or HIV status, which were defined as the set of predictor

variables. In our analysis, logistic regression model was used since our dependent variable is dichotomous (presence or absence of infection). Logistic regression coefficients were used to estimate odds ratios for each of the independent variables in the model and assess the chances of the variables being a 'risk factor'. All samples analysed in the three consecutive samples (n=758) were used. The independent variables identified for each analysis were:

Age (mothers or children)

HIV status (infected or uninfected).

Visit (Examination 1,2,3)

Village (total of seven villages were surveyed)

3.9.1 Results: Logistic Regression Analysis

The logistic regression model was used initially with all covariants for all data. The analysis showed age was a significant risk factor in most parasites except *T. trichiura* (OR 1.411, 95% CI 0.579 - 3.443; *p*=0.449) and *A. lumbricoides* (OR 1.312, 95% CI 0.899 - 1.916). Overall, there was no risk in relation to HIV status for infection with any parasites in the overall analysis. The analysis showed there was an increased risk to infection due to village of residence for infections with *E. coli* (OR 3.08 95%CI 1.344-7.055 *p*=0.008 for Nyahera village), *E nana* (OR 5.08 95% CI 1.104-23.35 *p*=0.04 for Khunyangu, and OR 7.17 95% CI 1.55-33.12 *p*=0.012 for Matayos, and OR 7.57 95% CI 1.59-36.1 *p*=0.011 for Usigu). The risk of infection with *A. lumbricoides* was also higher for residents in Siaya and Usigu villages (OR 0.418 95% CI 0.201-0.869 *p*=0.02; OR 0.476 95% CI 0.232-0.976 *p*=0.43 respectively). The results also revealed an increased risk to *B. hominis* infection associated with the

period of examination (OR 0.423 95% CI 0.223-0.804 *p*= 0.009). These risk were observed in the overall data independent of the ages of the participants. The data was consequently analysed within each age group and the results are presented below.

3.9.1.1 Risk assessment to parasite infection in mothers

Regression analysis revealed that mothers in some villages were at a higher risk of infection with *E. coli*, *E. nana* and *A. lumbricoides.* Infection with *B. hominis* varied by specific visit as shown on Table 3.13 below.

Mothers in Nyahera and Khunyangu were at a higher risk of infection with *E. coli* and *E. nana* (p = 0.011 and p = 0.044) respectively. Those in Siaya were also at a higher risk of infection with the same parasites (p = 0.004 and p = 0.026). There was also an increased predisposition to infection with *A. lumbricoides* for mothers in Nyahera, Siaya and Usigu (p = 0.021; p = 0.008; p = 0.008 respectively). The results showed there was an increased risk to infection with *B. hominis* in all mothers surveyed in the third examination (OR 0.376, 95% CI 0.183-0.770; p = 0.008).

Table 3.12 Potential risk of infections in mothers

Parasite	Variable	Odds Ratio	95% CI Lower limit	95% CI Upper limit	*p*-value
E. coli	Khunyangu	2.434	1.224	4.839	0.011
	Siaya	2.949	1.416	6.141	0.004
E. nana	Khunyangu	4.881	1.046	22.767	0.044
	Siaya	6.009	1.274	28.339	0.026
	Usigu	6.135	1.243	30.278	0.026
A. lumbricoides	Nyahera	0.386	0.172	0.865	0.021
	Siaya	0.311	0.136	0.707	0.008
	Usigu	0.313	0.132	0.742	0.008
B. hominis	Exam 3	0.376	0.183	0.770	0.008
All Parasitism	Khunyangu	3.831	1.316	11.147	0.014
	Siaya	2.917	1.087	7.826	0.033
	HIV +	0.664	0.363	1.214	0.184

Values generated by Logistic regression = binary logistic (categorical variables); (SPSS).

Table 3.12 shows the significant predictor variables ($p>0.05$) that showed increased risk to the respective parasite infection in mothers. Note HIV infection was not a risk factor to parasite infection in mothers.

Fig 3.13 Risk Estimate to parasitism in mothers by HIV status

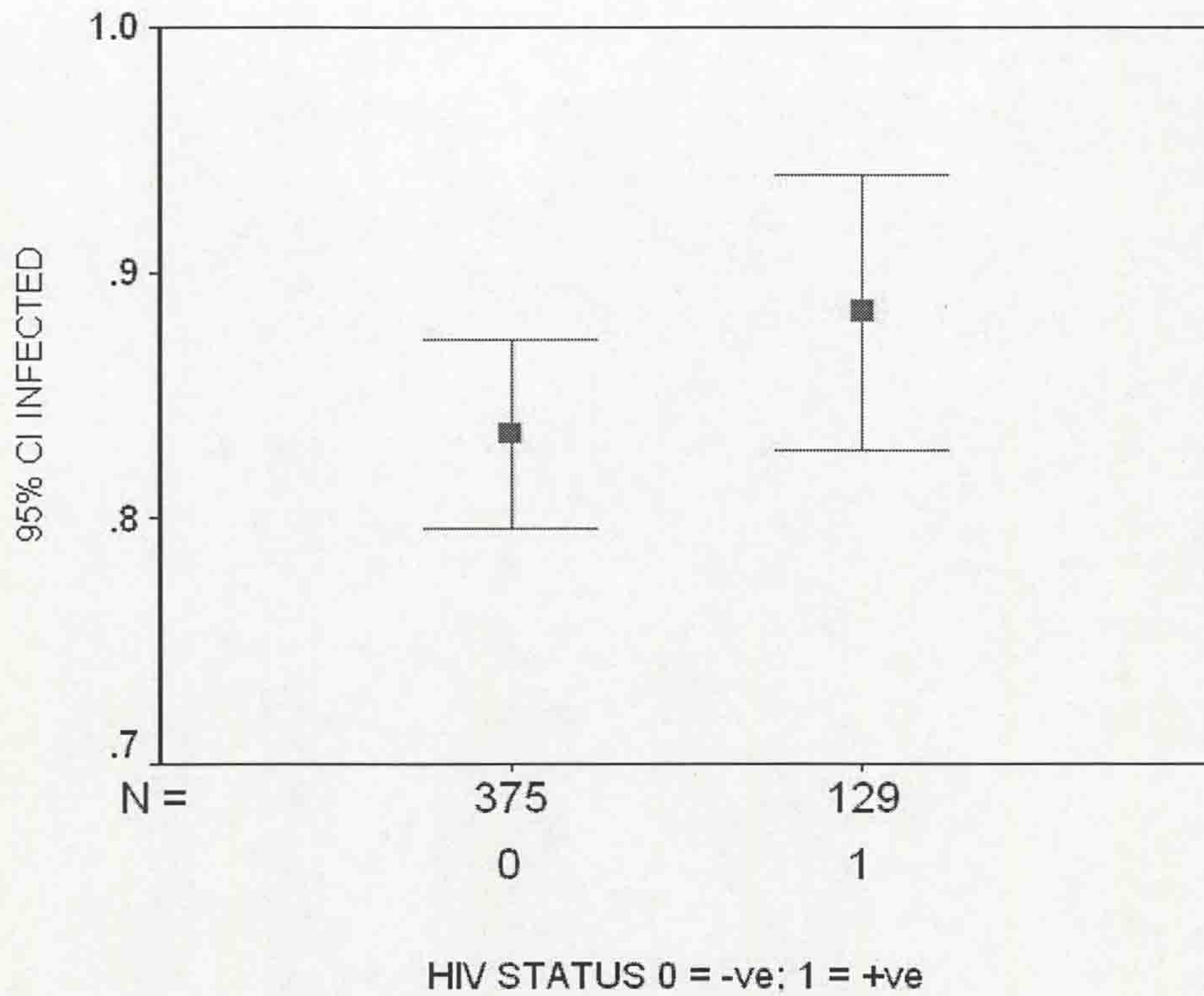


Fig 3.13 shows the estimated risk (confidence intervals for probability of having any parasitic infection). There was no difference in the risk of parasite infection due to HIV infection in mothers OR 1.505 (95% CI 0.823-2.752; p=0.115).

3.9.1.2 Potential risk factors of parasite infection in children

A similar analysis was done for children in the study. Children were separated from mothers in order identify any effects of the HIV status parasites that would be associated with. Results from the regression analysis revealed there were no significant risks to infection among children by Examination 1,2 or 3 (p=0.153, p=0.216, p=0.633 respectively) or by the seven village of residence (p=0.747, p=0.565, p=0.947, p=0.519, p=0.593, p=0.649, p=0.849 villages 1-7 respectively). HIV-infected children were however at a significantly higher risk to hookworm infection than HIV-uninfected children. Since there were 3 consecutive examinations, those found infected more than once were recorded as positive only once, while those recorded negative in more than one examination were recorded negative only once. This resulted to 115 children recorded as negative for hookwork infection for the three examinations while 38 were positive in the same period. The results showed there was an increased risk to hookworm infection in HIV-infected children (OR 2.333, 95% CI 1.046 - 5.203; $p = 0.032$) as illustrated by the Fig. 3.14 below.

Fig 3.14 Risk Estimate for hookworm infection in children by HIV status.

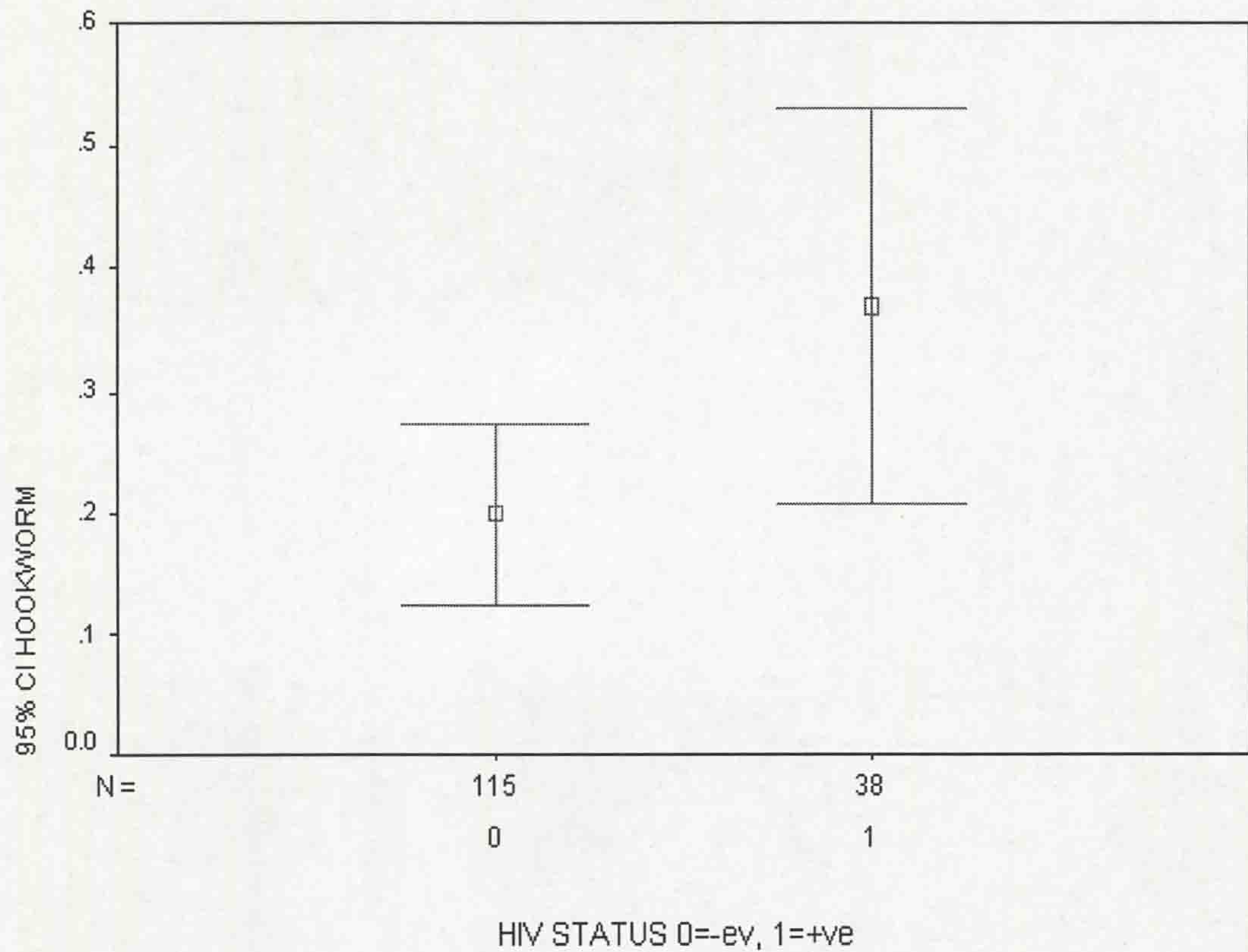


Fig 3.14 shows the estimated risk to hookworm infection in children (confidence intervals) by HIV status. The results confirmed that HIV infection predisposed the children to hookworm infections (OR 2.333, 95% CI 1.046 - 5.203; $p = 0.032$).

3.10 Discussion

Numerous studies on intestinal parasites have been undertaken in Kenya as summarised in the review by Chunge and others (1985). However, in the majority of the studies, relatively limited set of protozoa were found or recorded. Most targeted school children who are deemed as 'high risk' to intestinal parasittes. Moreover, the surveys were carried out before the current HIV/AIDS epidemic in the region. We therefore undertook to identify and follow the transmission patterns using prevalence data of these parasites in a community largely free of clinical disease in both mothers and children with or without HIV infection.

Parasite prevalence observed in the communities resembled those recorded in earlier studies in the area (Kinoti, 1971; Hall, 1981; Chunge *et al.*, 1985; Chunge *et al.*, 1989; Chunge *et al.*, 1991a). Infection rates as shown through prevalence data were similar for most parasites in the seven villages examined. Some increased risk of infection with *E. coli* and *E. nana* was identified in Khunyangu and Siaya villages. This is in spite of the fact that in 5/7 villages (including those with a higher risk), residents had access to piped water. The results confirmed that transmission levels for parasites with a faecal-oral route are still high in the villages. This is in agreement with an earlier observation that in many parts of Kenya, parasite infection rates are as high as they were in the 1940s despite the improved socio-economic status (Chunge *et al.*, 1985).

Parasite prevalence differed as expected by age, with mothers showing significantly higher infection rates than children. But infection with *A. lumbricoides* and *T.*

trichiura was similar in mothers and children. Our results also indicated that mothers in Nyahera, Siaya and Usigu villages were more predisposed to infection with *A. lumbricoides* than others. Except for Nyahera, the other two villages are situated closest to the lakeshore. It is therefore possible that there are differences in the dispersion and transmission of *A. lumbricoides* between villages.

An interesting observation was the high prevalence of *C. mesnili*. The organism is considered non-pathogenic in most cases with few studies investigating its presence (Chunge *et al.*, 1985). The highest prevalence similar to that observed in our study of around 17-20% was recorded in two other studies in central and eastern Kenya (Chunge *et al.*, 1991a; Chunge *et al.*, 1995). In their study, Chunge and others (1991a) observed that *C. mesnili* associated with persistent diarrhoea in children. Their study also noted a significant association of *B. hominis* to the occurrence of watery stool. In our study, a point prevalence of 13% and a consequent 3 and 6 months period prevalence of 21% and 28% respectively, were for *B. hominis*. Current reports suggest the parasite is associated with some gut pathology and modulates immune responses indicating it should be considered an opportunistic parasite (Gassama *et al.*, 2001). Other reports have shown increased frequency and severity of enteric disease in HIV-positive people with *B. hominis* infection (Llibre *et al.*, 1989; Cegielski *et al.*, 1993). Our results show that both *C. mesnili* and *B. hominis* occur frequently among the parasites fauna in mothers in the communities examined.

In our study, infection with *E. histolytica/E. dispar* was about 20% in all the villages. However, there were no significant reports of diarrhea, a situation that was

corroborated by the consistency of the faecal samples examined that were mostly well formed. This suggests that samples identified were most likely non-pathogenic *E. dispar*. There was also no difference in the occurrence of *E. histolytica/E. dispar* between the HIV-infected and un-infected persons. However, *E. histolytica* cysts have been identified in asymptomatic carriers making this diagnosis inconclusive (Tachibana *et al.*, 2000; Zaki *et al.*, 2001). It will therefore be important to differentiate the two species and assess their impact in relation to HIV/AIDS.

Different levels of intestinal parasites have been documented in HIV infected people with no clear and conclusive trend. While some reports show increased frequency in non-opportunisitic intestinal parasites (Aztori *et al.*, 1993; Tarimo *et al.*, 1996; Fontanet *et al.*, 2000a) others have reported either no increase (Wiwanitkit, 2001; Lebbad *et al.*, 2001) or even decrease in frequency (Lindo *et al.*, 1998; Anand *et al.*, 1998). Other studies have reported an increased prevalence of infection with amoebae in people with HIV infection (Cegielski, *et al.,* 1993; Fontanet *et al.*, 2000a). While HIV-infected mothers had significantly higher prevalence with *G. lamblia* and *B. hominis* in Exam 1, a risk assessment showed they were no more likely to be infected with the other different parasites than HIV-uninfected mothers. Since our communities were largely free of clinical AIDS, it is possible that interaction between lumenal parasites such as *A. lumbricoides* or non-invasive protozoa, and, the human immunodeficiency virus' at this stage does not lead to any observable changes in the hosts susceptibility or establishment that would present as changes in prevalence of the former.

A significant finding of the effects of HIV infection was observed in the case of children. HIV infection did not appear to be a risk factor in all the other parasites both helminths and protozoa. The risk to hookworm infection did not vary by village of residence or specific survey, only by HIV status. It was also not apparent in mothers. Our results confirmed that HIV-infected children were at a significantly higher risk to hookworm infections than HIV-uninfected ones ($p = 0.005$). This is significant as the area is endemic with hookworms and an increasing number of children are born with HIV-infection (Songok *et al.*, 2001). In our study, almost a third of the children were HIV-infected. The role of hookworm infections in the morbidity associated with HIV infection warrants further investigations

4.0 CHAPTER 4: CRUDE INCIDENCES AND CLEARANCE RATES

4.1 Introduction

In chapter 3, the prevalence of different parasites identified in the communities were profiled. An interesting finding was that parasites with similar routes of transmission (such as *E. coli, C. mesnili, B. hominis*) showed different prevalence. While some of the differences may be due to difficulties in detection, parasite incidence/clearance rates are also important factors. Another reason is different durations of infection for the specific parasites. The next section therefore deals with incidence, clearance rates and duration of infections for specific parasites from data accrued from the follow-up study in both mothers and children

4.2 Materials and Methods

The follow-up examinations allowed the computation of crude incidence and clearance rates in both mothers and children for the first and second survey.

In total, 149 participants were seen in both Examination 1 and 2. This included 98 (65.7%) mothers and 51 (34.2%) children. Because of the relatively small number of the people attending both examinations and the four categories required in the computation, for incidence and clearance rates analysis was done for each parasite in mothers and children regardless of the HIV status.

4.3 Results Three Months Crude Incidence and Clearance Rates

4.3.1 Crude Incidence and Clearance Rates in Mothers

Data from the first and second examinations was used to work out the crude incidence and clearance rates in mothers.

Codes for each category	
Total initially negative	= -
Became infected	= -+
Total initially infected	= +
Became uninfected	= +-

Table 4.1 Three months Crude Incidence and Clearance rates in Mothers

	-	-+	+	+-	Incidence %	Clearance rate %
Gl	94	6	4	3	6.4	75.0
Cm	83	20	15	10	24.1	66.7
Ec	41	20	57	13	**48.8**	22.8
Eh	84	18	14	10	21.4	71.4
En	92	20	6	3	21.7	50.0
Ib	88	9	10	5	10.2	50.0
Bh	92	9	6	5	9.8	83.3
Tt	93	8	5	5	8.6	100.0
Hw	79	16	19	10	20.3	52.6
Al	68	15	30	18	22.1	60.0

Numbers in bold show where incidence is greater than clearance rate.

Table 4.1 shows the 3 months incidence and clearance rates for each parasite among mothers (both with or without HIV infection). *E. coli* showed the highest incidence with levels twice as high as clearance rates for the same period. In all the other parasites, 3-months clearance rates was higher than incidence with some like *T. trichiura* recording 100% clearance rate. Similar analyses of three months incidence and clearance rates were done for the children.

4.3.2 Crude Incidence and Clearance Rates in Children

Codes: Exam 1 and 2

Total initially negative	= -
Became infected	= -+
Total initially infected	= +
Became uninfected	= +-

Table 4.2 Crude incidence and clearance rates in children

Parasite	-	-+	++	+-	Incidence %	Clearance rate %
Gl	42	9	9	6	21.4	66.7
Cm	44	6	7	5	13.6	71.4
Ec	39	19	12	5	48.7	41.7
Eh	45	4	6	5	8.9	83.3
En	51	8	0	0	15.7	84.3
Ib	47	2	4	1	4.3	25.0
Bh	51	5	0	0	9.8	0
Tt	49	2	2	1	4.1	50.0
Hw	47	10	4	0	21.3	0
Al	40	6	11	7	15.0	63.6

Table 4.2 shows the crude 3 months incidence and clearance rates for children (both HIV-infected and uninfected). A three months crude incidence for *G. lamblia* of 21.4% and a clearance rate 66.7% was recorded. This clearance rate was three times higher than incidence rate in the same period. Incidence and clearance rates for the same period for *E. coli* were 48.7% and 41.7% respectively. This would mean in the three months period only a marginal rise in prevalence for *E. coli* would have been observed in children since incidence and clearance rates were almost similar. However, point and period prevalence Chapter 3 showed there was a rise in infection with *E. coli* over the three and six months period. As in the case of mothers above (Table 4.2), most parasites showed a clearance rate much higher than incidence, suggesting parasite prevalence was falling over time. It was not possible to compute the clearance rates for hookworms and *B. hominis* due to insufficient counts.

4.3.3 Summary of Results

In summary, the direct measure of crude incidence and clearance rates does not take into account any infection gained and lost, or lost and regained during the 3 months observation period as it is based on the results observed at each end of the survey. In mothers *E. coli*, *C. mesnili*, *E. nana*, which have similar transmission routes showed relatively similar crude incidence and clearance rates (except for *E. coli*). Yet, the parasites presented different prevalence rates in mothers with *C. mesnili* at 20%, while *E. nana* was 11.9%, and *E. coli* had 62.7% (Fig 3.1). The prevalence results suggested other factors other than transmission routes determine the prevalence of these organisms in the community.

In order to account for these differences in prevalence another method was used to estimate the durations of infections for each parasite over the three and six months observation period.

4.4 Estimation of Transitions Rates using Bekessy's Model

Principle

Incidence and recovery rates can be estimated using a model described by Bekessy and others, (1976). The model describes the simultaneous estimation of incidence and recovery rates from a longitudinal study of positive and negative infections among a defined cohort allowing for several transitions between consecutive surveys. Identified persons are examined at defined intervals and their parasitism state determined at each survey using a standard diagnostic method.

4.4.1 Materials and Methods

Only participants seen in each consecutive examination are included in the estimates. A 2X2 table illustrating how numbers for examination were derived is shown on Appendix 3. The model is based on two proportions:

α = proportion of parasite positives at the second survey, in those negative at the first

β = proportion of parasite negatives at the second survey in those positive at the first

$$\alpha = \frac{\text{Number of positives at the second examination}}{\text{Total number of negatives in the first examination}}$$

$$\beta = \frac{\text{Number negatives at the second examination}}{\text{Total number positives in the first examination}}$$

The model assumes that in a given populations at a given time, there are only two disease states, either negative or positive for the infection. In this stochastic process, negatives become positive at a constant rate (h) and positive become negative at a constant rate (r). The model also assumes that the probability of making one transition from negative to positive or from positive to negative in any one time unit (a day) is small and that of

making two or more transitions in that time unit is negligible. The transition rates h and r are thereafter estimated ($\hat{h}$, $\check{r}$) from the transition frequency α and β in the following formula:

Formula 1

$$\hat{h} = \frac{\alpha}{t(\alpha + \beta)} \ln \frac{1}{1 - (\alpha + \beta)}$$

$$\check{r} = \frac{\beta}{t(\alpha + \beta)} \ln \frac{1}{1 - (\alpha + \beta)}$$

where t = time interval between surveys (in days for the estimation of daily rates)

The rates can only be estimated when $(\alpha + \beta) < 1$. If $(\alpha + \beta) \geq 1$, it means either the process was a non- Markovian[1] or the parameters were not constant between successive observations. The reciprocal of daily recovery rates ($1/\check{r}$) gives the expected duration of a positive episode or the duration of an infection. Duration of infection relates to prevalence of the disease, as an infection with a long duration is likely to be detected at any one time, thus will have a higher prevalence. A high incidence will also result in higher prevalence of the disease even with shorter duration of infection. A change in the two factors therefore, ultimately affect prevalence in a population as:

Prevalence = Incidence x duration of Infection.

[1]'Markov-chain' is a state in which the transition from one state to another is assumed to be independent of previous transitions, (Thrusfield, 1986)

4.4.2 Estimation of Daily Transitions in Mothers and Children

The number of mothers at different states of parasitism in examination 1 and 2 were summarized into four groups that included

Key:

- \- - = those negative in the first and second examination for each parasite
- \- + = those negative in the first exam and positive in the second
- \+ + = those positive in the first and the second examination
- \+ - = those positive in the first examination and negative in the second

Table 4.3 Counts for mothers examined in Exam 1 and 2

	Number of Mothers (N) in Examination 1 and 2				
	Time days	- -	- +	+ +	+ -
EC	90	21	20	44	13
EH	90	66	18	4	10
GL	90	88	6	1	3
CM	90	63	20	5	10
BH	90	83	9	1	5
IB	90	79	9	5	5
AL	90	53	15	12	18
HW	90	63	16	9	10

Table 4.3 shows the number of observed cases in mothers in each infection category of two consecutive examinations in the first 3 months. There were approximately ninety days between Exam 1 and 2. Using the Formula 1 stated above, the daily transition rates for incidence and clearance rates were calculated as shown in the Table 4.4 below

Table 4.4 Transition Frequencies for Mothers in Exam 1 and 2

Parasite	t = days	α= N-+/N-	β = N+-/N+
EC	90	20/41	13/57
EH	90	18/84	10/14
GL	90	6/94	3/4
CM	90	20/83	10/15
BH	90	9/92	5/6
IB	90	7/88	5/10
AL	90	15/68	18/30
HW	90	16/79	10/19

Transition rates were estimated for the individual parasites except *E. nana* and *T. trichiura*, which had insufficient, counts in some of the categories.

Table 4.5 Daily Transition rates in mothers

	Daily incidence & clearance rates for mothers in Exam 1 and 2			
	$\hat{h}$	$1/\hat{h}$	$\check{r}$	$1/\check{r}$
GL	0.00147	680	0.01720	58
CM	0.00700	142	0.01950	51
EC	0.00953	105	0.00445	225
EH	0.00680	147	0.01480	68
IB	0.00132	757	0.00830	120
BH	0.00313	319	0.02661	38
HW	0.00404	248	0.01047	95
AL	0.00514	194	0.01340	72

$\hat{h}$ daily incidence rate
$1/\hat{h}$ expected duration of a negative episode
$\check{r}$ daily recovery rate
$1/\check{r}$ expected duration of a positive episode

Table 4.5 shows the daily transition rates for the individual parasites in mothers. *Giardia lamblia* showed a daily estimated incidence of 0.00147, and a daily recovery rate of 0.0172.The daily clearance rate (recovery) was 11 x higher than the daily incidence (acquisition) while the estimated duration of infection was 58 days. The high parasite clearance, low daily incidence rate coupled with a short duration of infection would be reflected in a low prevalence for *G. lamblia* in mothers. An estimated 'parasite free' period of 680 days meant that mothers would have a long period between infections.

Entamoeba coli, the most common protozoa among mothers had an estimated daily incidence of 0.00953 and a daily recovery rate of 0.00445. The daily incidence was 2.14 times higher than daily recovery. This was the only parasite that recorded a higher daily incidence than daily clearance rate. Duration of infection was estimated at 225 days. The resulting high parasite acquisition and long duration of infections would result in an overall high prevalence for *E. coli.* This indicated for *E. coli,* apart from higher daily incidence the parasites' long duration would mean a high prevalence due to persistent infections in those infected. This was in contrast to the situation with *G. lamblia* that suggested a more rapid clearance of infection in fewer individuals within the same period. An estimated 105 days 'parasite free' period for *E. coli* would mean mothers were infected for longer periods than they were free of the infection.

With *C. mesnili,* estimated daily incidence was 0.007 while daily recovery rate was 0.0195, which was 2.7 times higher than the daily incidence. *Chilomastix mesnili* had an estimated duration of infection of 51 days; almost similar to that seen in *G. lamblia.* However, *C. mesnili* had a lower clearance rate resulting to a higher prevalence than *G. lamblia*. A 142 days 'parasite free' period combined with the short estimated duration of 51 days would mean the parasites' prevalence would remain lower that that of *E. coli* but higher than *G. lamblia*.

A similar summary was done for the children in the same period.

Table 4.6 Estimation of daily incidence and recovery rates for children

		Number of Children (N) in examination 1 and 2			
		- -	- +	+ +	+ -
	Time in days				
GL	90	33	9	3	6
CM	90	38	6	2	5
EC	90	20	19	7	5
EH	90	41	4	1	5
BH	90	46	5	0	0
IB	90	45	2	3	1
TT	90	47	2	1	1
HW	90	37	10	0	4
AL	90	34	6	4	7

Table 4.6 shows the number of observed cases among children in Exam 1 and 2. There were insufficient number of infected children in some of the categories limiting the analysis. Estimates for daily incidence and clearance rates were computed for the parasites where numbers were sufficient (i.e. without zero counts in any of the categories) as shown in Fig 4.7 below.

Table 4.7 Transition Frequencies for children in Exam 1 and 2

Parasite	= days	α= N-+/N-	β = N+-/N+
GL	90	9/42	6/9
CM	90	6/44	5/7
EC	90	19/39	5/12
EH	90	4/45	5/6
IB	90	2/47	1/4
TT	90	2/49	1/2
HW	90	4/10	-
AL	90	6/40	7/11

Table 4.8 Estimated daily incidence and clearance rates for children

	$\hat{h}$	$1/\hat{h}$	$\check{r}$	$1/\check{r}$
GL	0.00575	174	0.01791	56
CM	0.00339	295	0.01776	56
EC	0.01396	72	0.01195	84
EH	0.00274	365	0.0256	39
IB	0.00056	1786	0.0033	304
TT	0.000652	1533	0.01306	125
AL	0.00327	306	0.0138	72

Table 4.8 shows the daily transition rates in the individual parasites in children. *Giardia lamblia* showed an estimated daily incidence of 0.00575 and a daily recovery rate of 0.01791. This clearance rate was 3 times higher than the daily acquisition rate (incidence). Apparently, the daily recovery rate and the duration of infection of 56 days were similar to those observed in mothers (Table 4.5) but the children had a much higher daily incidence rate. An estimated 174 days of 'parasite free' period was however shorter than that observed in mothers. The lower clearance rate and higher incidence would result to children having a higher prevalence of *G. lamblia* than mothers even with similar duration of infection. This corresponded to the observed higher *G. lamblia* prevalence seen in children compared to mothers.

4.4.3 Summary of Results

Entamoeba coli had a much shorter duration of infection in children at only 84 days while daily incidence and daily recovery rates were almost equal (0.014 vs 0.012 respectively). The equally short duration of 'parasite free' period (72 days) meant re-infection with *E. coli* was high in children. In spite of almost equal rates of incidence and clearance, high prevalence would still be observed in children due to high re-infections. In the case of mothers, high prevalence with *E. coli* appeared to be due to the longer duration of infections.

In summary, our results were able to relate to differences in specific parasites to their different durations of infections. This was especially possible for parasites with similar transmission routes. However, the Bekessys model is based on the assumption that parasite detectability is 100%. In our study only one sample per person was examined (at least 3 times) and although our prevalence data is comparable to those from other surveys in the area, they are still likely to be underestimations of actual prevalence. An evaluation of the model was therefore, undertaken to assess the reliability of the model in estimation of transition rates assuming higher parasite detectability. The assessment is presented in the next section.

4.5 Evaluation of Diagnostic Tests in the Application of Bekessy's Model

The estimates using Bekessy's model presumes the diagnostic method is sensitive enough to detect all infections and clearance during the surveys. Since this was not achieved, an attempt to correct for low rate of detection was made through several adjustments of assumed proportions of false negative observations. This was worked

out by assuming several increases in number of positives observed through adjusted proportions as shown in Appendix 3.

‘Corrected’ daily transitions for duration and clearance rates were then computed as before. Since this was to assess the reliability of the model used, it was applied only for *E. coli*, a parasite that was most prevalent in mothers and children, and *G. lamblia* that was more prevalent in children and significantly lower in mothers. The proportions of adjustment and resulting numbers of ‘corrected’ observed counts are as shown in Tables 4.9-4.12 below. Corrected daily transition rates are also shown.

Table 4.9 Adjusted levels of transitions for *G. lamblia* in mothers

	Adjustment levels for *G. lamblia* in mothers				
Transition	Unadjusted	**0.9**	**0.7**	**0.5**	**0.2**
- -	88	71.3	43.1	22	3.5
- +	6	13.3	22.7	25	15.3
+ -	3	10.6	20.6	23.5	14.7
+ +	1	2.8	11.6	27.5	64.5
$\hat{h}$	0.0015	0.0052	0.018	0.0295	$(\alpha + \beta) \geq 1$
$\check{r}$	0.172	0.027	0.0303	0.0256	
$1/\hat{h}$	681	191	57	34	
$1/\check{r}$	58	37	33	39	

Numbers in bold show the adjustment levels
$\hat{h}$ = estimated daily incidence rate
$\check{r}$ = estimated daily clearance rate
$1/\hat{h}$ = estimated parasite free episode
$1/\check{r}$ = estimated duration of infection

Table 4.9 shows the adjusted rates for *G. lamblia* in mothers. The smallest change in parasite detection (0.9 where 1/10 negative cases were assumed to have been positive) resulted to a significant increase in estimated daily incidence and subsequent drop in 'parasite free' period from 681 days to 191 days. This was in contrast with the insignificant change observed in the estimated daily clearance rate that resulted to a moderate change in duration of infection from 58-37 days. The duration of infection remained stable for the other adjustments including when 50% of the negative cases were assumed to have been positive. No estimates were done for the 0.2 adjustments due to model violations as $\alpha + \beta \geq 1$.

Table 4.10 Adjusted levels of transitions for *G. lamblia* in children

	Adjustment levels for *G. lamblia* in children				
Transition	Unadjusted	**0.9**	**0.7**	**0.5**	**0.2**
--	33	26.7	16.2	8.3	1.32
-+	9	11.1	16.8	12.8	7.1
+-	6	8.4	11.3	11.5	6.5
++	3	4.8	10.5	18.6	36.1
$\hat{h}$	0.00575	0.009133	0.01498	0.0292	0.04987
$\check{r}$	0.01791	0.01976	0.01573	0.0184	0.009
$1/\hat{h}$	174	109	67	34	20
$1/\check{r}$	56	51	64	54	111

Numbers in bold show the adjustment levels
$\hat{h}$ = estimated daily incidence rate
$\check{r}$ = estimated daily clearance rate
$1/\hat{h}$ = estimated parasite free episode
$1/\check{r}$ = estimated duration of infection

Table 4.10 shows the adjusted rates for *G. lamblia* in children. This time the increase in the estimated daily incidence was not as high as that seen in mothers. The change was less for the estimated daily clearance rate that resulted to almost no significant change in the duration of infection. The estimated duration of infection remained almost unchanged up to the 0.5 adjustment level where the estimated daily clearance rate dropped resulting to a significant increase in duration of infection.

Table 4.11 Adjusted levels of transitions for *E. coli* in mothers

	Adjustment levels for *E. coli* in mothers				
Transition	Unadjusted	**0.9**	**0.7**	**0.5**	**0.2**
--	21	17.01	10.29	5.25	0.84
-+	20	19.9	18.41	15.25	7.36
+-	13	13.4	13.51	11.75	5.96
++	44	47.7	55.79	65.75	83.84
$\hat{h}$	0.00953	0.0112	0.01543	0.0209	0.0334
$\check{r}$	0.00445	0.00455	0.00466	0.00427	0.00266
$1/\hat{h}$	105	89	65	48	30
$1/\check{r}$	225	220	217	234	376

Numbers in bold show the adjustment levels

$\hat{h}$ = estimated daily incidence rate
$\check{r}$ = estimated daily clearance rate
$1/\hat{h}$ = estimated parasite free episode
$1/\check{r}$ = estimated duration of infection

Table 4.11 shows the adjusted rates for *E. coli* in mothers. The minimum adjustment (0.9) resulted to only a modest increase in the estimated daily incidence and a consequently minimal reduction in the 'parasite free' period. The adjustment resulted to a minor increase in the estimated daily clearance rate (0.00445 to 0.00455) that resulted to an equally small drop in the estimated duration of infection (225-220 days). This trend remained in the other two adjustments. However, at the highest adjustment (0.2, assuming 8/10 negative cases were actually positive), there was drop in the estimated daily clearance that resulted to large increase in the estimated duration of infection (234 to 376 days).

Table 4.12 Adjusted levels of transitions for *E.coli* in children

	Adjustment levels for *E. coli* in children				
Transition	Unadjusted	**0.9**	**0.7**	**0.5**	**0.2**
--	20	16.2	9.8	5	0.8
-+	19	18.9	17.5	14.5	7
+-	5	6.3	7.7	7.5	4.2
++	7	9.6	17.5	24	39
ĥ	0.01396	0.0174	0.0249	0.0334	0.0513
ř	0.01195	0.0127	0.01263	0.0107	0.00556
1/ ĥ	72	57.5	40.2	30	19.5
1/ř	84	78.7	79.2	93.7	180

Numbers in bold show the adjustment levels

ĥ = estimated daily incidence rate
ř = estimated daily clearance rate
1/ ĥ = estimated parasite free episode
1/ř = estimated duration of infection

Table 4.12 shows similar adjustments and resulting changes in transition rates for *E. coli* in children. While the estimated daily clearance showed minimum decrease in the first two adjustments (0.9 and 0.7), there was a shift to an decrease in the clearance rates observed at the 0.5 and 0.2 adjustment that led to significant increases in the estimated duration of infections (from 94 to 180 days). A steady increase in estimated daily incidence resulted to a corresponding drop in the 'parasite free' periods

The overall trends in the duration of infection are shown in Fig 4.1 and Fig 4.2 below.

Fig 4.1 Trends on estimated duration of infections for *E. coli* at different adjustment levels in mothers and children

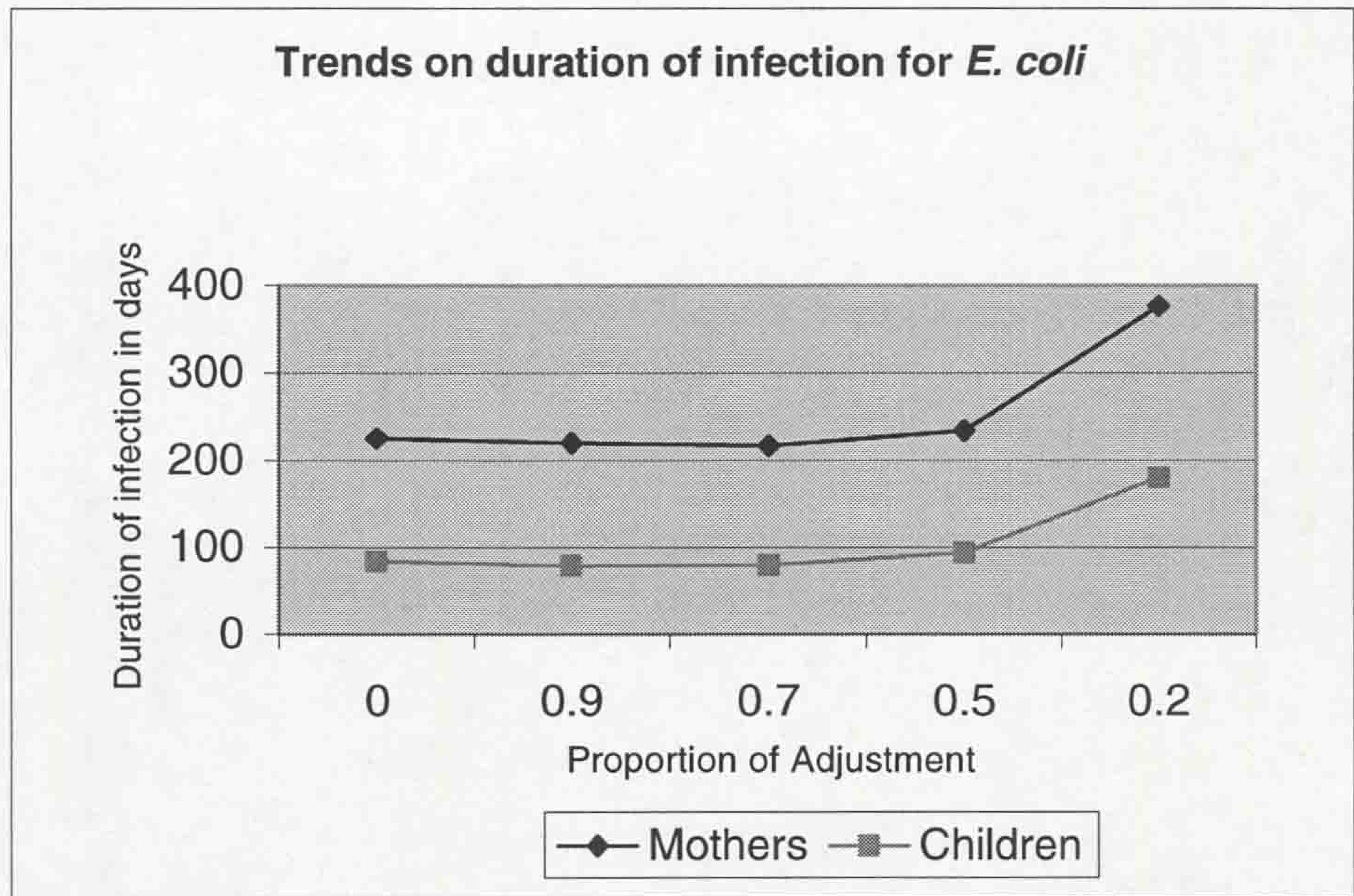


Fig 4.1 shows the trends of duration of infection at different adjustments on parasite detection levels. Note the almost unchanged duration of infections in both mothers and children even where 5/10 negatives are presumed positive.

Fig 4.2 Trends on estimated duration of infections for *G. lamblia* at different adjustment levels in mothers and children

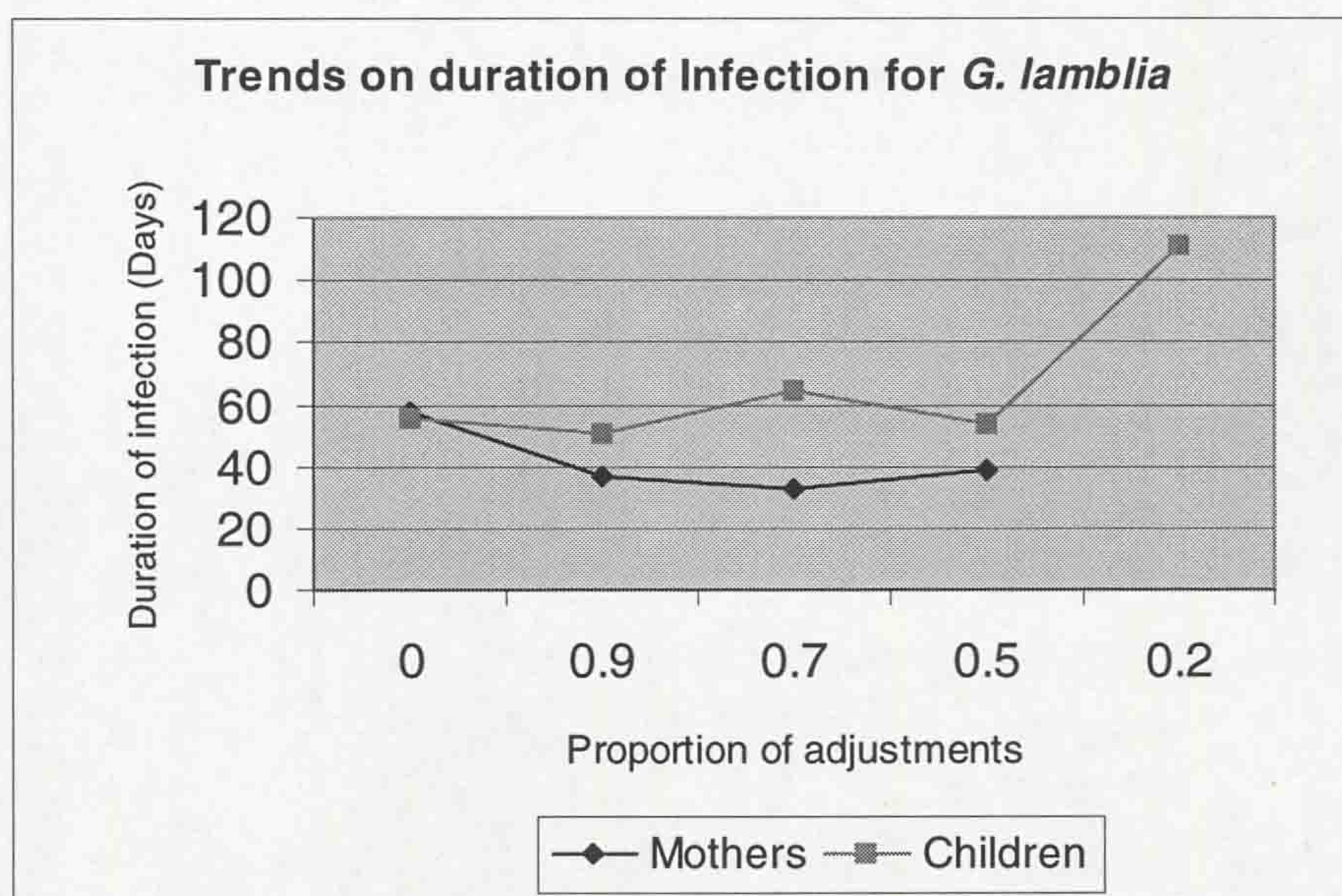


Fig. 4.2 shows the trends in duration of infection for *G. lamblia* at different adjustment levels. The trend was again stable for adjustments up to the 0.5 level when a sudden increase in duration was observed.

4.6 Discussion

Our results on prevalence of different parasites had revealed their differences in infection rates within the communities and between mothers and children. Prevalence data on *E. coli* suggested environmental contamination and transmission was high for parasites with the faecal-oral route. This would mean that the level of exposure would be fairly similar for most parasites in the communities. While numerous factors affect the hosts' susceptibility and establishment of parasitic infections, there is less information on the effect of different durations of infections on the overall parasite prevalence.

The crude incidence and clearance rates indicated a markedly higher rate of clearance for most parasites compared to acquisition. This would have resulted in a decline in parasite prevalence over time, which was not the case. The observation did not tally with the steady or increased levels of parasite prevalence observed in the three surveys described in Chapter 3. Since prevalence is a product of incidence and duration of infection, the direct crude incidence and clearance rates could be an imprecise way of assessing transmission mechanisms within the community. This was more pronounced in parasites that had a low prevalence such as *T. trichiura* where 100% clearance rates were recorded. Using the crude methods low detection is interpreted as either low incidence or high clearance rate. This analysis has limitations in that it does not take into account the daily and continuous transitions of parasitism from positive to negative and vice versa.

The model described by Bekessy and others (1976) estimates daily incidence, and daily clearance that can then be used to estimate duration of infection for each parasite. The model is reliable for short interval studies in a population homogenous in age and location as the two factors affect exposure and immunity. We considered our study communities fit for the application of the model since the two factors of age (mothers and children below 2 years of age) and environment (different villages) were fairly homogenous. Using this model the daily incidence and clearance rates for most parasites identified was analysed. The results showed *E. coli*, in mothers presenting the longest duration of infection (225 days) while it was shortest in *B. hominis*. In children, duration was longest for *I. buetschlii* and shortest for *E. histolytica*. Interestingly, *G. lamblia* and *C. mesnili* presented similar durations in mothers and children (between 51-58 days). These parasites have presumed similar transmission routes but present different prevalence in the community.

Our results revealed that specific parasites had different durations in mothers and in children that related to the differences observed in prevalence. This was apparent in the transmission estimates for *E. coli*. A long duration of infection of 225 days contrasted with a much shorter estimates in children (84 days). Assuming exposure was similar, disparity observed in the point prevalence would be a reflection of the differences in duration of infections. Still, a three and six months period prevalence suggested incidence was high in children (over 75% *E. coli* six months period prevalence in mothers and children). Estimated daily incidence and clearance rates were almost equal in children. This suggested that despite the shorter duration of infection in children, re-infection was prevalent, resulting to a high period

prevalence. In mothers the combination of high daily incidence rate and a longer duration of infection resulted to a persistently high *E. coli* prevalence. It also suggested that exposure does not decrease with age for protozoa with a faecal/oral route of transmission, as has been observed by others (Chunge *et al.*, 1991a; Ashford and Atkinson 1992).

Conversely, the lower estimated daily incidence for *G. lamblia* in mothers resulted to an equally low prevalence despite similar durations of infection with that in children (56 and 58 days respectively). Unlike the transmission estimates observed in *E. coli* infections, the results suggest the lower prevalence in mothers was due to the very low incidence compared to that in children. This was also apparent in the long 'parasite free' period (680 days) between infections. Infection with *G. lamblia* results in some age related protective immune responses (Garcia *et al.*, 1998). Such effects make our interpretation of transitions for *G. lamblia* difficult since the model assumes a 'Markovian' state of transition. An increase in immune responses in some individuals would mean previous exposures have an effect on consequent transition rates, a state that appear to be the case in mothers. Previous studies investigating duration of infections rely on frequent examination of faecal samples recording infection episosed and parasite clearance (Farthing *et al.*, 1986; Gilman *et al.*, 1988; Núñez *et al.*, 1999). Application of this method for *Giardia* infection in children showed diverse durations with Farthing and others (1986) recording durations of 2-6 weeks among children in Guatemala. Others reports estimated the duration of *Giardia* infection at 60 days in Peru (Gilman *et al.*, 1988) and 30 days in Egypt (Sullivan *et al.*, 1988). A duration of 131.7 +/- 26.5 days was reported in Kenya for

G. lamblia in children using Bekessy's model (Chunge *et al.*, 1991a; Chunge *et al.*, 1991b). These durations were comparable to our estimates of 56 and 58 days in mothers and children respectively.

Assessment of the model used in our study revealed there was little change in the duration of infections even when up to 50% of the negative cases were presumed to have actually been positive. Increasing detectability meant a presumably higher number of infected people were identified in the community (that is a decrease in false negatives). Yet, this increase in number of positives detected did not appear to affect the estimated duration of infections for the parasites. Routine detection methods are more likely to underestimate the occurrence of parasites, with almost 40% single stool examinations being false negatives (Nazer *et al.*, 1993; Hiatt *et al.*, 1995). Our findings are therefore significant in that they show the estimated transitions are reliable even where sensitivity of the tests used is low.

The effect of adjustments was however, different for the daily incidence and the consequent 'parasite free' period. Increasing the proportion of detectability assumed a higher number of positive cases in the communities. A slight increase in positive cases led to a large increase in incidence and an equally large drop in estimated 'parasite free period'. The results suggest that the model is sensitive to the detection test used as any false positive would reflect as an overestimation of daily incidence and therefore shorter 'parasite free period'. The effect was more pronounced where prevalence was low as in *G. lamblia* infections in mothers compared to that in children.

In conclusion, our results demonstrated the possible durations of infections of some of the parasites identified in the communities studied. While various factors including age, immune heterogeneity, and genetic factors may affect the infection rates in individuals within a community, our results show how some of the disparity in prevalence relates to differences in duration of infections for specific parasites. Assessment of the model showed that these transition estimates can be derived from prevalence data even where sensitivity of the test used is low. However, the model is also sentive to sample size where parasites with higher prevalence showed more robust estimates such as in *E. coli* compared to the large daily transition estimates observed where low counts were recorded.

PART 2: *CRYPTOSPORIDIUM*: IDENTIFICATION, GENOTYPING AND PHYLOGENY

5.0 CHAPTER 5: *CRYPTOSPORIDIUM* INTRODUCTION AND LITERATURE REVIEW

5.1 Introduction

This section covers the introduction of the second part of the study. This included the biology, epidemiology, and molecular analysis of *Cryptosporidium*. The results of the prevalence and genotype identification of *Cryptosporidium* parasites identified in the study are presented.

5.1.1 *Cryptosporidium*: General Introduction

Cryptosporidiosis in humans is a severe enteric disease of the immunocompromised as well as a major worldwide waterborne and zoonotic infection. The disease is largely attributed to *Cryptosporidium parvum* based on copro-diagnostic tests that rely on oocyst morphology. However most *Cryptosporidium* species and diverse genotypes are indistinguishable either in direct microscopy or by indirect fluorescence antibody techniques. Classification of *Cryptosporidium* species has been difficult and confusing as the definition of a species as a "reproductively isolated interbreeding population" appears inadequate for the species (Xiao *et al.*, 2000c). It is now clear that the predominant *Cryptosporidium species* infecting humans refers to two main genotypes namely, *C. parvum* "human" genotype (genotype 1) and *C. parvum* "bovine" genotype (genotype 2) that appear to have independent reproductive cycles (Morgan *et al.,* 1999e). The former is transmitted predominantly between humans while the latter constitutes the zoonotic *C. parvum* genotype 2, cycling between humans, domestic and wild mammals (Fayer *et al.*, 2000). Molecular analysis has led to identification of other *C. parvum* genotypes such as 'pig' type, 'mouse' type, 'monkey' type, 'ferret' type, and 'marsupial' type in the respective animals (Morgan

et al., 1999d). Others like *C. parvum* 'dog' type (now renamed *C. canis*), *C. felis* and *C. meleagridis* have been isolated from both immunocompromised and immunocompetent persons, apart from their natural hosts (Pieniazek *et al*., 1999; Morgan *et al*., 2000a; Alves *et al*., 2001; Guyot *et al*., 2001; Pedraza-Diaz *et al*., 2001a; Xiao *et al*., 2001a). The role of these novel species and genotypes in human cryptosporidiosis is still unclear. Their identification and the apparent increase in the incidence of the disease have put new challenges on epidemiology of cryptosporidiosis. The disease has long been recognised as a leading opportunistic infection in individuals infected with HIV/AIDS (Current *et al*., 1993). Being a zoonosis and waterborne, it is a health concern due to the risk to the general public.

In this regard species and strain identification through molecular techniques have gained a lot of interest lately as they offer precise identification of the *Cryptosporidium* species. However, to date only, a limited number of isolates have been fully characterised especially from HIV infected patients from developing countries. Our current study is on the genotypic identification of *Cryptosporidium* organisms recovered from different groups of people in Kenya, both HIV infected and un-infected people. Results were compared to those of isolates from other regions including Malawi, Brazil, Vietnam, Thailand and UK. Initial identification was by Kinyouns' Ziehl-Neelsen (ZN) staining and microscopy. Confirmation was by nested polymerase chain reaction (PCR) while genotyping was by restriction fragment length polymorphism (RFLP) of the hypervariable region of the 18S rRNA gene and confirmed by fragment sequencing.

5.2 History

Cryptosporidium (a Greek term meaning hidden spore) was first described by Tyzzer, (1907) from the gastric mucosa of asymptomatic mice, and the parasite was named *Cryptosporidium muris*. He observed the sexual and asexual cycle of the parasite and identified the presence of four naked sporozoites in the oocysts. Tyzzer (1912) identified further a different *Cryptosporidium* species that has smaller oocysts inhabiting the small intestines of the domestic mouse and named it *Cryptosporidium parvum.*

6.2.1 Taxonomy of *Cryptosporidium*

Cryptosporidium is a genus in the phylum Apicomplexa. The phylum includes a large group of sexually reproducing, spore forming protozoa with an apical complex at some stage in their life cycles (Dubey *et al.*, 1990). The term Apicomplexa was introduced to replace phylum Sporozoa, when parasites of the genus *Perkinsus* were classified together with sporozoans in the 1970s. Current phylogenetic evidence shows *Perkinsus* is more closely related to dinoflagellates than to Sporozoa, thus there has been suggestions that the term Sporozoa should be restored to refer to this phylum (Cox, 2002). Sporozoa, comprise a large number of parasites with over 4000 species known to date. It is estimated there could be over 2 million species including the coccidia in this phylum (Levine, 1985; Bush *et al.*, 2001). Coccidia develop in the gastrointestinal tract of vertebrates during part or whole of their life cycle. One group is capable of extra-intestinal asexual development and constitute the tissue cyst-forming coccidia that include the genera *Besnoitia, Sarcocystis*, *Neospora* and

Toxoplasma (Dubey *et al*., 1990). The other group develop only in the gastrointestinal or respiratory mucosa without formation of tissue cysts and include the genera, *Eimeria, Cyclospora, Isospora* and *Cryptosporidium*. Some animal hosts harbour numerous species of sporozoan parasites, each occupying a specific niche and infecting cell types within the gastrointestinal tract (Levine, 1982). Taxonomy of these species is based on a combination of morphological features from light and electron microscopy, life cycles, transmission and host specificity (Levine *et al*., 1980). Among the coccidian group, *Cryptosporidium* oocysts are among the species with the smallest exogenous stages and differences are more difficult to discern especially by light microscopy (Morgan *et al*., 1999d). *Cryptosporidium* is currently classified under the class Sporozoasida, subclass Coccidiasina, Order Eucoccidiorida, suborder Eimeriorina, Family Cryptosporidiidae (Levine, 1984).

Recent advances in phylogeny of protozoa have provided new insights into the genetic relatedness of many organisms warranting review of their taxonomy (Morgan *et al*., 1999d). This is most apparent in protozoa as organisms tend to be the 'exceptions' within the taxonomic rules derived for higher eukaryotes. The genus *Cryptosporidium* presents one of such dilemmas as new information challenges current classification among the coccidia. The proposed classifications show it is in the phylum Sporozoa, Class Coccidea, Order Eimeriida, suborder Eimeriorina, Family Cryptosporidiidae (Cox, 1998). Some molecular analysis and *in-vitro* studies have suggest the classification may still need further revision as the genus does not appear to belong to the suborder Eimeriorina like other coccidia (Barta, 1997; Zhu *et al*., 2000a).

5.2.2.1 *Cryptosporidium* species

Initially, over 23 species infecting more than 170 animals were described based on morphology and their natural hosts (Dubey *et al.*, 1990). However, experimental infections in animals, detailed morphology and isozyme analysis invalidated some after revealing similarities among the 23 species described, while others were found to be other coccidian (Levine and Corliss, 1980). This led to a revision of the taxonomy of *Cryptosporidium* genus that recognised eight major species (Levine, 1984). These comprised of *C. muris, C. parvum* in mammals (Tyzzer, 1910; 1912), *C. meleagridis, C. baileyi*, in avians (Slavin, 1955; Current and Upton *et al.*, 1986), *C. serpentis* infecting reptiles (Levine, 1980), *C. nasorum* in fish, while *C. wrairi* has been described in guinea pigs (Vetterling et al., 1971) and *C. felis* in cats (Iseki, 1979). An additional species, *C. saurophilum* has since been described in lizards, Schneider's skink (*Eumeces schneideri*) and in desert monitors (Koudela and Modry, 1998). Lately, the *Cryptosporidium muris* isolated from cattle has been shown to be a distinct species and named *C. andersoni* (Lindsay *et al.*, 2000; Sreter *et al.*, 2000). *Cryptosporidium canis* has also been described as a distinct species (Fayer *et al.*, 2001), bringing the current total of valid species to eleven. The number is bound to increase as more information becomes available on the numerous *C. parvum* cryptic species identified currently refered to as *C. parvum* 'marsupial' genotype, pig genotype, mouse genotype, kangaroo genotype and corvine genotype (Morgan *et al.*, 1999c; Morgan *et al.*, 1999d; Ong *et al.*, 2002; Xiao *et al.*, 2002,)

Among all the species in this genus, *C. parvum* has been most studied in biology, transmission and infectivity to both humans and animals (Levine; 1984; Dubey *et al.*,

1990; Fayer *et al.*, 1997; Tripori and Griffiths 1998). Thus, most of the information on cryptosporidiosis is based on data from this particular species. *Cryptosporidium parvum* has been isolated in over 79 mammalian species with varying virulence and infectivity. The organism has maintained its validity as a single species, with different genotypes and strains (O'Donoghue, 1995).

5.2.2 Life cycle and its significance

Unlike other coccidia, species of the genus *Cryptosporidium* have several unique characteristics. These include their relative lack of host specificity, resistance to most antimicrobial agents, and ability to autoinfection through the production of two types of oocysts. Additionally, they have a unique intra-cellular but extracytoplasmic location within the host cell. Recently, distinct extra-cellular trophozoite/gamont-like and gametocyst-like stages similar to those of the gregarines have been observed in *in vivo* and *in vitro* studies of *C. parvum* (Hijjawi *et al.*, 2001). Morphologically, *Cryptosporidium* parasites have the fewest and smallest naked sporozoites per oocyst among the coccidia (Dubey *et al.*, 1990). Consequently, these unique characteristics have added to the interest and urgency in clarification of its classification.

Cryptosporidium has a monoxenous life cycle that is completed in one host (Levine, 1984). It has six major developmental stages confined to the microvilli region of the hosts' epithelial cells (Fig 5.1). Oocysts once shed can survive in the environment for 2-6 months especially in cool moist places. On ingestion by suitable host, oocysts rupture to release sporozoites, which invade the epithelium of the small intestines and initiate the asexual reproduction. The extracellular forms (sporozoites, and two

generations of merozoites and microgametes) are highly vulnerable and have been shown to have a short life span *in vitro* . Under the protective mucus gel of the villi, they invade adjacent cells where internalisations is very rapid, a process rarely seen microscopically in *in vitro* studies (Tzipori and Griffiths, 1998)

Fig 5.1 *Cryptosporidium* life cycle

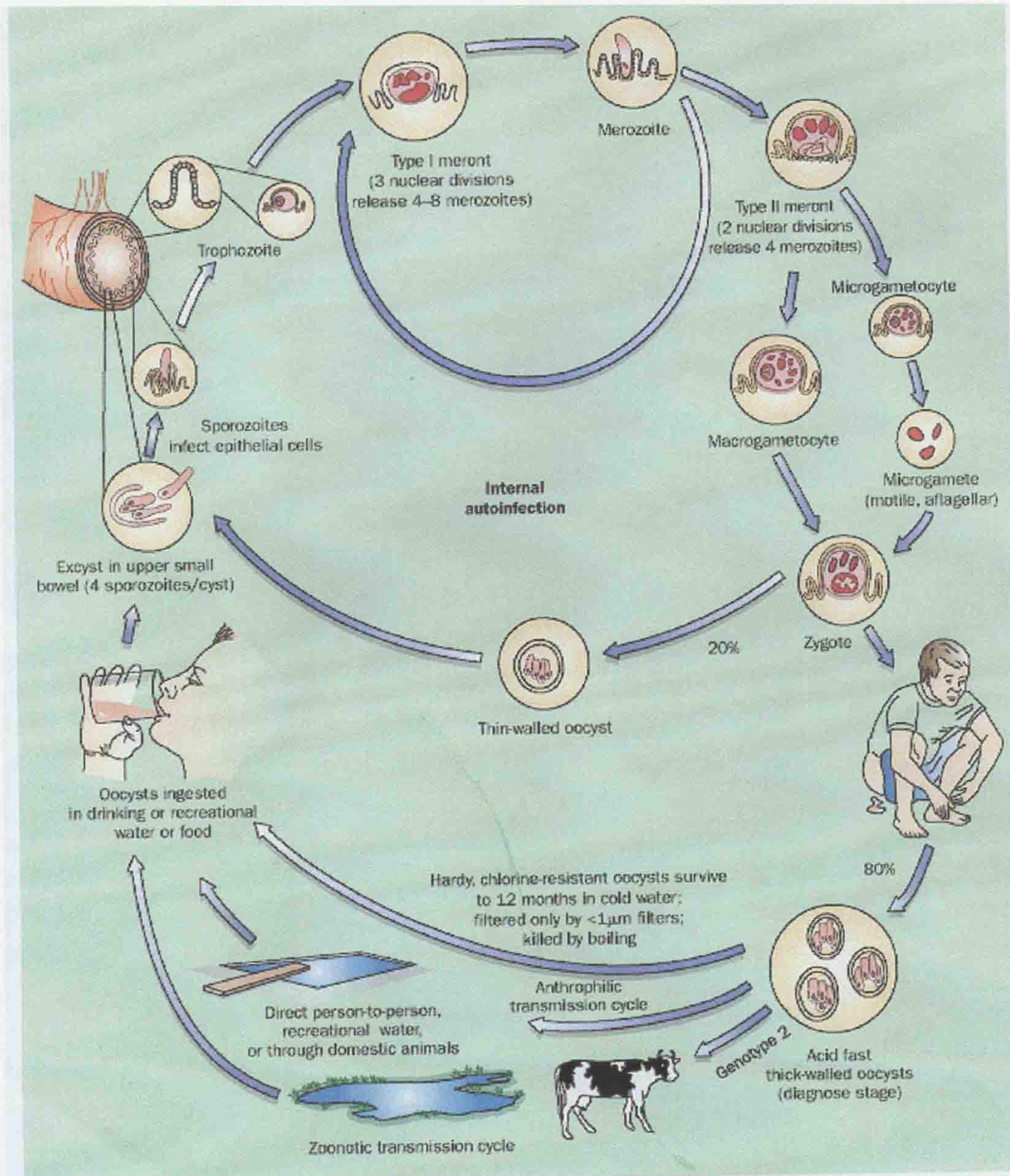


Fig 5.1 shows the life cycle of *Cryptosporidium*. Note the proposed separate transmission routes for the two *C. parvum* genotypes (1 and 2). Adapted from Kosek *et al*., 2001.

It has been suggested that the short extracellular span makes the organism elusive to a wide range of chemotherapeutic and immunotherapeutic agents. To be effective, such agents would need to be present in high concentrations, in all areas of the gut, where the organism is replicating (Tzipori and Griffiths, 1998). Such a scenario is unlikely due to the fluidity and dynamism of the gut flow especially when compounded by diarrhoea that results from the disruption of the mucosal function.

The sporozoites attach to the enterocyte and penetrate the cell wall becoming the trophozoites. Trophozoites are enclosed in a parasitophorous vacuole, a structure derived from the host cell, separate from the cytoplasm by a feeder organelle. This structure is unique to *Cryptosporidium* spp. The intracellular stages are well protected, and the developmental process takes about 8-12 hours. The longer duration at this stage offers a possible target for intracellular chemotherapy as is other coccidian parasites such as *Toxoplasma,* (Tzipori and Griffiths, 1998).

Two generations of asexual reproduction result in large numbers of meronts that re-infect neighbouring enterocytes. Thereafter, merozoites differentiate into micro- and macrogametes (sexual stage) that fertilize to produce zygotes. Subsequently, the latter mature forming the cyst wall, containing four naked sporozoites (Navin and Juranek, 1984; Upton and Current, 1985). Oocysts undergo sporulation while still within the intestinal brush border. Eighty per cent of the oocysts develop a thick wall while 20% have a thin wall. The latter can sporulate in the gut and re-infect the same host, a process that is unique to *Cryptosporidium*. The mechanism for internal sporulation and autoinfection appears to be modulated by the hosts' immune factors (Riggs,

1997). In cases of immunosuppression the production of thin walled oocysts is thought to be enhanced, resulting in increased mucosal damage and chronic diarrhoea. In immunocompetent hosts however, infection results to an apoptotic process that leads to death of infected cells and cessation of the infection. In chronic carriers, *Cryptosporidium* appears to modulate the hosts' tumour necrosis factor alpha (TNF-α) that initiates apoptosis, thereby establishing optimal growth and maturation with no apparent clinical damage to the hosts (McCole *et al.*, 2000).

Experimental passaging of *C. parvum* through different animals does not appear to alter the pathogenicity of the specific strain, remaining virulent (or avirulent) in the natural hosts (Tzipori, 1983). While oocysts remain viable after exposure to disinfectants such as sodium hypochlorite and sodium hydroxide, their infectivity is destroyed by ammonia, chlorine, formal-saline or freeze-drying conditions. Extreme conditions of high or low temperatures kill the oocysts but they can remain viable at -10^0 C for a week. Heating for up to 1 minute at 72^0 C renders them non-infectious (Fayer *et al.*, 1997).

5.3 Distribution of *Cryptosporidium*

Cryptosporidium has a worldwide distribution with low point prevalence but sporadic waterborne epidemics and person-to-person outbreaks (Tzipori, 1983). The first clinical case of cryptosporidiosis was described by Slavin (1955), in a turkey with severe diarrhoea infected with *C. meleagridis*. Thereafter cryptosporidiosis was reported in a wide range of young animals, including calves (Pohlenz *et al.*, 1978; Reese *et al.*, 1982; Anderson and Hall, 1982), lambs (Tzipori *et al.*, 1982; Anderson

BC, 1982), pigs (Moon *et al*., 1982), goats (Mason *et al*., 1981), chickens, (Dhillon *et al*., 1981), cats (Iseki, 1979), dogs and numerous wild animals (Dubey *et al*., 1990) including snakes, monkeys, parrots, and deer, (Navin and Juraneck 1984) and marsupials (Morgan *et al*., 1995).

Human cryptosporidiosis was first reported by Nime and others, (1976) in a young girl presenting an acute case of enterocolitis where *Cryptosporidium* was the only discernable pathogen. But it was not until 1982 that cryptosporidiosis as a significant human disease was noted by the Centers for Disease Control and Prevention (CDC), (1982). The disease was identified in 12 adult male homosexual patients presenting with overwhelming watery diarrhoea and all had advanced HIV/AIDS. Since then, it has been recognised that *Cryptosporidium* can cause an intractable cholera-like diarrhoea in immunocompromised patients worldwide (Blanshard *et al*., 1992; CDC, 1994; Fayer, *et al*., 1997; Tzipori and Griffiths, 1998). It is a major cause of severe diarrhoea in children and immunologically naive populations throughout the world (Bogaerts *et al.,* 1984; CDC, 1984; Hayes *et al*., 1989; Rahman, 1990; Moore *et al*., 1994; Kosek, 2001).

5.4 Epidemiology and Transmission

Cryptosporidiosis has been detected in over 170 different animal species including 77 mammalian. Most cases are attributed to different strains of *C. parvum* (Current 1985; Dubey *et al*., 1990; Fayer *et al*., 1997). Epidemiological determinants of the organism include its small size (can pass through ordinary water filters), low infectious dose, its high chlorine resistance, and its zoonotic potential (Kosek *et al*.,

2001). Oocysts are spread via faeces of infected humans or animals. Since 1993, *C. parvum* gained public attention as the most serious and difficult to control cause of waterborne-related diarrhoea in developed countries (Tzipori and Griffiths, 1998). Acute diarrhoeal outbreaks have been reported in domestic animals in herds in the United Kingdom (UK), the United States of America (USA), and Australia presenting as a serious highly contagious enteric disease in calves, lambs, piglets and goat kids (Tzipori, 1983). Transmission of *Cryptosporidium* through recreational and municipal drinking water has also been documented (Jokipii *et al.*, 1983; D'Antonio *et al.*, 1985; Atherton *et al.*, 1995; Furtado *et al.*, 1998; Hunter *et al.*, 2001). The Milwaukee (USA) outbreak in 1993 confirmed the epidemic potential of *Cryptosporidium* as a waterborne pathogen with more than 400,000 people becoming infected in this single incident (CDC 1993; Current, 1996). There are however, fewer reports of waterborne outbreaks from most countries than expected perhaps indicating under-detection and under-reporting, more than absence.

5.4.1 Incidence

Cryptosporidium ranks as one of the four most common aetiologies of diarrhoea being most common in infants but with episodic disease occurring throughout life (Fayer *et al.*, 1997). Most of the data are based on laboratory reports or hospital records and may therefore be biased; variations occur due to specimen selection criteria, diagnostic tests used, availability of facilities, and reporting systems. It is therefore difficult to ascertain incidence or prevalence worldwide although there is little doubt about the organisms' contribution to the burden of gastrointestinal disease (Casemore *et al.*, 1997). While point prevalence maybe low, it has a high life time

prevalence rate as theoretically everyone is at some risk of getting cryptosporidiosis due to its ubiquitous nature, wide range of animal reservoirs, and high infectivity (Tzipori and Griffiths, 1998).

Prevalence of infection among the immunocompetent ranges from 0.1-2% in developed countries to 0.5-10% in developing countries in both diarrheic and asymptomatic children. Sporadic reports however record high prevalence of over 30% (Casemore, 1990). Seroprevalence shows much higher rates ranging from 25-35% in developed countries and 42-57% in developing countries like China and Brazil and 64% in Latin America (Ungar, 1988; Casemore, 1990; Zu *et al.*, 1994; O'Donoghue, 1995;). Differences in disease prevalence could be due to genuine geographic variations, demographic and social economic factors, timing of surveys especially in short duration studies or methodology factors (Casemore *et al.,* 1997). High rates of asymptomatic infections probably reflect hyper-endemicity and persistent re-infections (Casemore *et al.*, 1997). Peak incidence is usually in 1-5 year olds, initial exposure is early in developing countries but peak prevalence is similar in developed and developing countries perhaps due to the children's higher attendance to day care centres in the former (Clavel *et al.*, 1996; Fayer *et al.*, 1997). The prevalence is also higher in adults under 45 years than older ones perhaps due to their child caring activities that may expose them to oocysts, or, increased acquired age related immunity in older people (Casemore *et al.,* 1997). Studies analysing antibody titers in the USA showed seropositivity of 13% in under 5 year olds, 38% in those aged 5-13 yrs and 58% in adolescents (Fayer *et al.*, 1997). In Brazil, exposure was much earlier with almost all children under 2 years being seropositive while in Anhui, rural China,

only half of the 5-7 year olds were seropositive while 75% of the 11-13 year olds had been exposed (Kuhls *et al.*, 1994; Zu *et al.*, 1994). The differences could be due to the environmental factors such as water sources and level of hygiene.

5.4.2 Risk factors

Apart from immunosuppression, malnutrition appears to be a risk factor to cryptosporidiosis perhaps due to the effect of nutrition on immunity leading to an increase in susceptibility. A study on children in Mexico showed a 40% higher risk of the disease among poor urban children compared to 22% among rural children (Miller *et al.*, 1994). Viral infections such as measles have also been shown to increase susceptibility or increase the severity of cryptosporidiosis in children (Groves *et al.*, 1994).

Transmission is seasonal and higher in warm and wet climates or seasons. However, local factors like farming activities and endemic strains in each region also influence transmission. In Spain, cryptosporidiosis among children was highest in autumn-winter periods perhaps due to more attendance at day care centres (Clavel *et al.*, 1996). In the UK *Campylobacter* is the most commonly identified enteric pathogen but in certain times of the year, *Cryptosporidium* in the most common enteric pathogen in 1-5 year old children (McLauchlin *et al.,* 2000).

Infection and subsequent disease development is also linked to the specific *Cryptosporidium* species or strains in the environment or source of infection. Experimental studies have show different strains have different viability, virulence

and minimum infective dose (Okhuysen *et al*., 1999). Well-adapted strains may require very low infective doses of oocysts but may present a less virulent disease in terms of incubation period, clinical severity and secondary transmissions (Chappell *et al*., 1996). Other less adapted isolates or primary animal strains may require a higher minimum infective dose but may produce more serious disease (Casemore *et al*., 1997).

5.5 Transmission

Transmission patterns vary from region to region perhaps due to a multiplicity of factors and interactions between the parasites, hosts, and environments (Casemore, 1990; Fayer *et. al,* 1997). Until recently, many laboratories did not routinely check for *Cryptosporidium* organisms in cases of diarrhoea, thus it is often under-reported and misdiagnosed (Skeels *et al*., 1990). Generally, transmission is highest at the start of rainy seasons when survival of the oocysts and dissemination is easier. In the UK peaks of incidence are in spring and late autumn to early winter coinciding with peak rainfall and increased farm activities (Casemore, 1990; Clavel *et al*, 1996; Hart 1999). Outbreaks or sporadic cases, however, continue to appear in different parts of the UK, mostly around the same time each year but reasons for these patterns are not clear. Different parts of the country tend to have infections from the same strains with the southern more densely populated area mainly recording the human-to human strains (genotype 1) while the northern parts of UK records more of the zoonotic strains (genotype 2) (Furtado *et al*., 1998; McLauchlin *et al*., 1998; Hart, 1999).

5.5.1 Zoonotic transmission

Initial studies of human infections traced the source of cryptosporidiosis to calves (Current, 1985; Fayer *et al.*, 1986). Since then, cross transmission studies using this strain, between humans and a variety of other animals have been demonstrated. The zoonotic *C. parvum* 'bovine' genotype has been identified in different wildlife where a sylvatic cycle is well maintained (Fayer *et al.*, 1997; Perz and Le Blancq, 2001). Lately, there have been reports of other zoonotic species such as *C. muris, C. meleagridis, C. felis* and *C. canis* infecting humans (Morgan *et al.*, 2000a; Xiao *et al.*, 2001a; Pedraza-Diaz *et al.*, 2001a; Gatei *et al.*, 2002; Ong *et al.*, 2002). While it is clear these species are of animal origin, their transmission routes are not yet documented.

Cryptosporidium is suspected to be a major cause of transient diarrhoea among animal handlers (Moon and Woomansee, 1986; Rahman *et al.*, 1996), including veterinarians (Anderson and Donndelinger *et al.*, 1982). Infected animals excrete large numbers of oocysts sometimes up to 10^{10}daily for up to two weeks after the disappearance of symptoms (Tzipori, 1982; Orihel and Ash, 1995). However, despite the continuous presence of pets in households, rarely are they implicated as a source of infection. The presence of oocysts in the pets may imply that both owner and pet have a common reservoir source (Casemore *et al.*, 1997). Genotype analysis enables identification of strains in circulation and determines their possible sources may transmission routes (Morgan *et al.*, 1999d).

5.5.2 Person to person

Cryptosporidiosis is a disease of public health concern due to the direct person-to-person transmission either directly or via faecal-oral contamination. Person to person transmission has been proposed in outbreaks occurring in day-care centres (CDC, 1984), homosexual transmission (Ma and Soave 1983; Goodgame *et al.,* 1993), nosocomial infections (Koch *et al.*, 1985) and household cross-infections (Fayer *et al.*, 1997). In endemic areas person-to-person transmission through poor hygiene standards, perpetuates cryptosporidiosis in the communities (Reese *et al.*, 1982; Hunt *et al.*, 1984; Casemore, 1990; Rahman *et al.*, 1990). In the USA, *Cryptosporidium* has been implicated in many day-care centres outbreaks and was isolated in 65% of the cases from an outbreak in Pennsylvania, 55% in Michigan, and 17% in Georgia (CDC, 1984). A survey in Georgia day care centres showed cryptosporidiosis is nearly endemic (Addis *et al.*, 1991). Other outbreaks have been reported in Spain, Portugal and South Africa (Walters *et al.*, 1988; Melo *et al*, 1988; Clavel *et al.*, 1996). Sporadic cases are often associated with recent diarrhoea or contacts with asymptomatic carriers (Current *et al.*, 1983; Current *et al.*, 1994; Rahman *et al.*, 1996).

5.5.3 Food borne transmission

Epidemiological evidence from UK has shown that consumption of specific foods such as raw milk, offal, and raw fresh sausages is associated with *Cryptosporidium* infection (Casemore, 1990). Infection is most common in naïve persons with no previous cryptosporidiosis exposure (Laberge *et al.*, 1996). Elsewhere, a study in Guinea Bissau showed an increased risk of infection from the consumption of stored

cooked food (Casemore *et al.,* 1997). Documentation of food as a source of infection has been difficult due to limitations in sensitivity of diagnostic tests, while tracing sources of infection can be hard due to delay in disease development (Casemore *et al.,* 1997).

Few cases however, have been traced and fully reported. In the USA, 26% of party-goers fell ill after consumption of freshly pressed apple cider (Millard *et al*., 1994). In the outbreak, 33 cases were confirmed by identification of oocysts while 127 became clinically ill. Pre-patent period averaged 6 days ranging from 1-13 days. Of the clinically sick, 82% experienced diarrhoea and vomiting while 16% had vomiting without diarrhoea. Oocysts were recovered from the remaining cider and from faecal samples from a calf in one of the farms that supplied the apples. Primary infections showed severe symptoms compared to consequent secondary infections, which had longer incubation periods of 1-24 days with an average of 8 days, and only 60% of the patients had vomiting. This suggested that initial exposures were with a high dose of the organism resulting in more severe disease than the consequent secondary infection through human-to-human transmissions (Casemore *et al.,* 1997).

Mechanical transmission by cockroaches, flies and dung beetles has also been documented, which increase the risk from food-borne contamination (Zerpa and Huicho, 1994; Graczyk *et al*., 1999; Mathison and Ditrich, 1999).

5.5.4 Nosocomial infections

Cross infections from patient to patient, and between patients and hospital or caring staff members have been recorded (Koch *et al.*, 1985; Sarabia-Arce *et al.*, 1990; Casemore *et al*, 1994; Weber *et al.*, 2001). In one of the early documented cases of nosocomial infections in Denmark, 18 of HIV-infected patients in a hospital as well as the worker and a visitor to the unit contracted cryptosporidiosis (Ravn *et al.*, 1991). Eight out of the 18 patients later died of the disease. Infection was thought to originate from one of the patients probably via a contaminated ice-making machine.

Nosocomial transmissions are possible because of the low infectious dose and the parasite's resistance to disinfectants and anti-microbials. An outbreak among patients in a renal unit in Argentina resulted in 11 out of 14 patients and one staff member contracting cryptosporidiosis from one infected patient (Roncoroni *et al.*, 1989). In the UK, there have been reports of leukemic children contracting the cryptosporidiosis in hospital, while in North Wales in an infectious disease unit, five nurses contracted cryptosporidiosis from a HIV-infected patient (Foot *et al.*, 1991; Casemore *et al.*, 1994). In the USA two outbreaks occurred in hospital day care centres while 28 children in a hospital in Portugal contracted cryptosporidiosis within a day care centre (Combee *et al.*, 1986; Melo *et al*, 1988).

5.5. 4 Travellers' diarrhoea

Cryptosporidium is one of the parasites implicated as a cause of travellers' diarrhoea along with other protozoa such as *Giardia* and *Cyclospora* (Fayer *et al.*, 1986; Crawford and Vermund, 1988; Ungar, 1990; O'Donoghue 1995). These organisms

occur frequently in travellers as mixed infections suggesting a common epidemiology. In some areas, exposure to livestock during farm visits is significant while international travel especially to developing countries increase the risk to infection (Casemore *et al.*, 1997; Fayer *et al.*, 1997).

5.6 Waterborne transmission and outbreaks

The biggest impact of *Cryptosporidium* as a public health concern has been on the water supply and resource industry (Moore *et al.,* 1994). Surveys in the USA showed 65-97% of all surface water contained *Cryptosporidium* oocysts (USA Gov-Environmental Protection Agency- EPA-website; 2001). While *Cryptosporidium* organism has been implicated in waterborne outbreaks, few studies have characterised the precise species isolated (Kramer *et al.*, 1996). So far, 'human' and 'bovine' and 'cervine' genotypes of *C. parvum* and *C. meleagridis* have been identified contaminating water for human consumption (Xiao *et al.*, 2000a; Glaberman *et al.*, 2002).

Important sources of *Cryptosporidium* oocysts are discharges of untreated and treated domestic sewage and agricultural run-off (Medema and Schijven, 2001). Contamination occurs in surface water from catchments sources or boreholes through ground seepage. Major waterborne outbreaks are thought to be of zoonotic origin. However molecular analysis confirmed that the largest waterborne outbreak recorded in Milwaukee was due to the 'human' type *of C. parvum* probably due to sewer contamination of the municipal water sources (Cicirello *et al.*, 1997).

Since 1984, waterborne outbreaks have been reported in USA, UK, Japan and Australia, France, Sweden, Zambia and South Africa among other areas (Fayer *et al.*, 1997). In the USA, all outbreaks of waterborne cryptosporidiosis detected from 1984 to 1993 occurred in communities where water utilities met state and federal quality standards for acceptable drinking water. In all incidences, all surface water supplies implicated had been filtered. These outbreaks indicate that water treatment standards did not adequately protect against waterborne cryptosporidiosis (CDC, 1995). Similar outbreaks in the UK have been linked to inadequately treated drinking water as well as contaminated swimming pools due to faecal accidents in recreational waters (Hunt *et al.*, 1984; Evans *et al.*, 1996; Furtado *et al.*, 1998; Hunter *et al.*, 2001).

Recently, major resources have been allocated to ensure that all water for human consumption is free of *Cryptosporidium* and other protozoa such as *Giardia* in UK and USA (UK- Drinking Water Inspectorate, (DWI website), 2001; USA- Environmental Protection Agency - EPA-website; 2001). In the UK, current guidelines require water supply organisations ensure less than 1 oocyst per 10L of water. Control strategies are aimed at removing or killing *Cryptosporidium* oocysts and *Giardia* cysts. Multiple barriers are designed to remove or inactivate the pathogens. Water treatments method such as sweep flocculation engulfment, interaction of oocysts with alum, ultraviolet and other radiations has been developed to rid water of *Cryptosporidium* (Bustamante *et al.*, 2001; Craik *et al.*, 2001). Water decontamination through parasite inactivation using ozone and monochloramine is applied especially where contamination is with faecal material such as in swimming pools (Driedger *et al.*, 2001; Hirata *et al.*, 2001). Stringent measures including flow

cytometry, PCR, electrorotation, ELISA, turbidity correlation, colorimetric methods and immunofluorensce are undertaken to sample and analyse water quality and ensure compliance with these standards (Bustamante *et al.*, 2001).

However, these processes are expensive and may not always ensure safe water. A recent cost-benefit assessment estimated that the prevention of one reported case of cryptosporidiosis costs roughly UK£1000 (Fairley *et al.*, 1999a). While stringent measures might ensure drinking and utility water is largely free of *Cryptosporidium* at the point of supply, they do not prevent sporadic cases of cryptosporidiosis from post water-treatment contamination. Nor can they guarantee complete removal of oocysts. These measures are therefore set as a regulation and not based on quantified health risk (DWI, 2001). In other countries including some states in USA and Canada, treatment and monitoring for cryptosporidiosis is less stringent (Wallis *et al.*, 1996; Payment *et al.*, 2000)

Monitoring the water supply may not predict an outbreak or rule out source of infection as the distribution of oocysts is not homogenous and varies from source, treatment and distribution points (Casemore *et al.,* 1997). Waterborne cryptosporidiosis has been linked to treated borehole and ground water (Bridgman *et al.*, 1995; Willocks *et al.*, 1998). Very low doses detected at any point may miss out non-homogenous clusters of oocysts from boluses of contaminated water. In general, the greater the number of oocysts, the greater the chance of a significant number of consumers being exposed to infectious doses (Casemore, 1992; Casemore *et al.*, 1997). Water related factors include abnormal weather conditions like flooding that

increase surface water runoff, contaminating water sources while high turbidity reduces water treatment efficiency. Other factors include ground water seepage into reservoirs, or burst underground water and sewer pipes (Casemore *et al.*, 1997).

Although reports of waterborne outbreaks in developing countries are few, this is more likely due to under-reporting, misdiagnosis or complete absence of conclusive investigations of such outbreaks (Fayer *et al.*, 1997). Waterborne outbreaks have been documented in Lusaka, Zambia where over 500 people presented with diarrhoea lasting over 2 weeks (Kelly *et al.*, 1997). Sustainable control measures to prevent waterborne cryptosporidiosis will need to be based on local conditions and needs.

5.7 Epidemiology in People with HIV-infection

In immunocompromised patients cryptosporidiosis presents as a chronic life threatening cholera- like infection that is characterised by severe diarrhoea, extreme weight-loss and abdominal cramps (Kamel *et al*, 1994; Robinson, 1995; Chintu *et al*, 1995). Some reports of the occurrence of *Cryptosporidium* infection in association with HIV in some African countries are listed in Table 5.1. Prevalence ranges from 3-4% in western countries to about 50% in hospital patients in developing countries. A study in Kenya (Mwachari *et al.*, 1998) indicated that detection of C*ryptosporidium* oocysts was the single most significant predictor of death in HIV infected persons. In a study in Bamako Mali, cryptosporidiosis was associated with chronic diarrhoea, dehydration and emaciation resulting in over 40% mortality (Pichard *et al.*, 1990).

However, chronic diarrhoea without cryptosporidiosis is also common. In one study of 217 HIV adult patients in Abidjan, 8.7% were infected with *Cryptosporidium*, yet,

78.9% of the patients had chronic diarrhoea, 89.4% had abdominal pain and 94.7% had weight loss (Assoumou *et al.*, 1993). Clinical cryptosporidiosis is more severe among children or immunocompromised people. A study among South African children admitted in a hospital showed a prevalence of 11.9% and a case fatality rate of 22.6% (van den Ende, 1986). Still, most clinical laboratories do not process stool specimens for *Cryptosporidium.* Where diagnosis is undertaken, tests are mainly based on morphology of oocysts, tests that do not distinguish the different species and genotypes of the parasite that infect humans (Morgan *et al.*, 2000b). These limitations makes it difficult to map out the epidemiology of *Cryptosporidium* infections or correlate the severity of disease to specific species and strains so far identified as in humans.

Table 5.1 Studies on *Cryptosporidium* infections in HIV-infected and non-infected persons in Africa

Country	Cohort	Prevalence	Reference
DR Congo	endoscopy-patients	30%	Colebunders *et al.*, 1988
Rwanda	Hospital patients	16.2%	Kadende *et al.*, 1989
Bamako, Mali	"	38%	Pichard *et al*, 1990
Abidjan, Cote d'Vore	"	8.7%	Assoumou *et al.*, 1993
Dakar, Senegal	"	13.9%	Dieng *et al.*, 1994
Ethiopia	"	39.7%	Mengesha, 1994
Lusaka Zambia	Children (1.5-5yrs)	14%	Chintu *et al.*, 1995
Younde Cameroon	Patients 2-52 yrs	15.8%	Same-Ekobo, *et al.*, 1997
Tanzania	Hospital patients	56% seroprevalence Specific IgG	Gomez *et al.*, 1995
Addis Ababa, Ethiopia	Hospital patients	25.9%	Fisseha *et al.*, 1998
Nairobi, Kenya	Adults cohort	17%	Mwachari *et al.*, 1998
Guinea-Bissau	HIV-2 patients	11%	Lebbad *et al*, 2000
Studies in HIV uninfected children			
South Africa	Children hospital	11.9-15% < 2yrs	van den Ende, 1986
South Africa	Day care centre	73% outbreak	Walters *et al.*, 1988
Kenya	Children <5yrs	3.8%	Simwa *et al.*, 1989
Guinea Bissau	Children	6-17.6% seasonal	Molbak *et al.*, 1990
Gabon	Children <2yrs	3.1% formed stool 24% diarrhea cases	Duong *et al.*, 1991
Sudan	Children hospital	7.8%	Hassan *et al.*, 1991
Enugu, Nigeria	School children	39.5% diarrhea 24.2% formed stool	Okafor and Okunji, 1996
Burkina Faso	Children <36months	5.2%	Narco *et al.*, 1998

5.7.1 Clinical manifestation and Treatment

5.7.1.1 Clinical symptoms

Cryptosporidium has a prepatent period of 2-13 days with a mean of 9 days and a median of 6.5 days. This has also been corroborated *in vitro* life cycles, animal models or healthy volunteers (Current, 1985; Current and Reese, *et al.,* 1986; Gut *et al.*, 1991; Chappell *et al.*, 1996). Clinical manifestation is largely dependent on host and type of infection (outbreak or sporadic). In outbreaks or experimental infections patients present with illness characterised by diarrhoea, abdominal cramps, anorexia, fever and vomiting (MacKenzie *et al.*, 1995, Chappell *et al.*, 1999). In about 20% of such infections, patients experience recurrence that may be mild within 1 week for up to 3 months.

Similar symptoms are observed in sporadic cases in children and infants. In some areas of the tropics where cryptosporidiosis is endemic, there is growing evidence that the disease has a long term effect observed as shortfalls in linear growth and weight gain (Checkley *et al.*, 1997; Checkley *et al.*, 1998; Agnew *et al.*, 1998). Studies in children in Brazil and Peru indicate even asymptomatic infections are associated with long-term growth deficits (Guerrant *et al.*, 1999). The studies showed infected children had long periods of diarrhoeal illness with some occurring intermittently for up to 21 months and accompanying fitness deficits. In Guinea Bissau, cryptosporidiosis in infants was associated with an increase in all-cause mortality that was independent of differences in socio-economic status, breastfeeding practices or nutritional status (Mølbak *et al.*, 1993).

Respiratory cryptosporidiosis is thought to be common though in apparent. A study in Ivory Coast revealed about a fifth to a third of children with cryptosporidiosis had pulmonary symptoms (Kone *et al.*, 1992). Among children admitted to hospital with enteric disease, about three times as many of those with cryptosporidiosis have pulmonary symptoms compared to those with other gut pathogens (Sallon *et al.*, 1991). When respiratory cryptosporidiosis occurs, patients present with croupy cough, wheezing and dyspnoea. In such cases oocysts are detectable in sputum, tracheal aspirates, broncho-alveolar lavage or alveolar exudates (Forgacs *et al.*, 1983; Kocoshis *et al.*, 1984). It is not clear whether the oocysts are a result of replication in the bronchial epithelium or as a result of emesis. The current identification of human infections with zoonotic species has raised the question of their pathogenesis. As yet, it is not clear what the predilection sites of species such as *C. baileyi*, *C. meleagridis* or *C. muris* in humans are.

In immunocompromised patients, clinical signs range from transient diarrhoea to profuse cholera-like enteric disease with resulting high mortality (Crawford and Vermund, 1988; Ungar, 1990; Blanshard *et al.*, 1992). Transient disease or asymptomatic carriage is common in people with CD4 counts of greater than 250 X 10^6 /L (Blanshard *et al.*, 1992). Patients with CD4 counts less than 50 X 10^6/L present with profuse cholera like illness and have a greater risk of disseminated cryptosporidiosis in other organs such as liver and the central nervous system (Pitlik *et al.*, 1983; Blumberg *et al.*, 1984; Brady *et al.*, 1984; Vakil *et al.*, 1996).

In enteric disease, the main pathology is the erosion of the intestinal epithelia leading to severe malabsorption as the parasites propagate in the brush border (Simon *et al.*, 1994). Profuse diarrhoea leads to hypovolemia, shock, and severe electrolyte imbalance (Blanshard *et al.*, 1992). A stool frequency of up to 7L per day with a loss of up to 17 litres of bodily fluid has been recorded (Ungar, 1990). The pathogenesis of this phenomenon is not well understood as no toxin has been isolated and the actual disruption of the intestinal mucosa does not match the severe clinical picture (Blanshard *et al.*, 1992; Moore *et al.*, 1994; Guarino *et al.*, 1994; Griffiths, *et al.*, 1998).

Survival rate at one-year post infection in patients with chronic diarrhoea is significantly shorter (60%) than those with transient symptoms (90%) (Sanchez-Mejorada and Ponce-de-Leon, 1994). In severe cases, infections may result in the colonisation of the pancreatic duct epithelium and the gall bladder (Hawkins *et al.*, 1987). The resulting infection carries a poor prognosis as seen among some patients in the Milwaukee outbreak (Kramer *et al.*, 1996). Biliary carriage is common in HIV patients that are less immunocompromised and may be significant for the relapse of cryptosporidiosis seen in chronic disease (Casemore *et al.,* 1997). In most natural infections, oocyst shedding occurs intermittently suggesting that screening tests should review at least 3 separate stool examinations to avoid false negative results (Crawford and Vermund, 1988; Garcia-Rodriguez *et al.*, 1990, Arrowood, 1997). Association between intensity of oocyst excretion and symptoms is not clear, although diarrhoeic patients shed more oocysts than those with enteric symptoms without diarrhoea (Chappell *et al.*, 1996).

5.7.1.2 Cryptosporidiosis treatment

No consistently effective treatment for cryptosporidiosis has been found. Patient management involves reducing loss of fluids in case of diarrhoea and boosting the immune systems. The drugs currently indicated in the treatment for cryptosporidiosis give unpredictable outcomes. Double blind control studies on the treatment of diarrhoea caused by *C. parvum* using nitazoxanide in AIDS patients resulted in the resolution of diarrhoea in 3-4 days and the cessation of oocysts shedding (Rossignol *et al.*, 1999; Rossignol *et al.*, 2001). Conversely, another study evaluating the efficacy of paromomycin, azithromycin, and nitazoxanide administered to patients with disseminated cryptosporidiosis showed the drugs were no more effective than placebo (Hewitt *et al.*, 2000; Giacometti *et al.*, 2001).

A recent review suggests nitazoxanide has a high potential for treatment of cryptosporidiosis and as a broad-spectrum anti-protozoa agent (Gilles and Hoffman, 2002). There are suggestions that the differences in responses to treatment is linked to the infective *Cryptosporidium* strain or genotype. A recent report on a patient treated with paromomycin showed effective parasite clearance and resolution of the diarrhoea despite the patient being heavily immunocompromised (Caccio *et al.*, 2002). Other studies propose that *Cryptosporidium* might have some distinct genotypic characteristics that reflect in the different phenotypic profiles some of which may confer resistance to most anti-coccidial drugs (Zhu *et al.,* 2000a; Zhu *et al.,* 2000b).

So far, however, it is clear that a competent immune system is the major factor in resolving cryptosporidiosis. Where patients are co-infected with HIV, antiviral

treatments including highly active anti-retroviral treatment (HAART) regimes or unspecific immunomodulators coupled with antimotility agents offer the best results against the disease (Tzipori and Griffiths, 1998; Schmidt *et al.*, 2001).

5.8 Other *Cryptosporidium* species isolated in humans

Lately, species other than *C. parvum* are increasingly being isolated from humans (Morgan *et al.*, 1999e). This has been partly due to increased interest in the parasite and improved diagnosis using techniques able to distinguish the different species and genotypes of the organism. However, it is clear there is increase in incidence of *Cryptosporidium* infections especially in immunosuppressed populations (Fayer *et al.*, 2000). Several species and genotypes have so far been documented infecting humans.

5.8.1 *Cryptosporidium meleagridis* and *C. baileyi*

Cryptosporidium meleagridis was first described by Slavin from turkey poults presenting with severe diarrhoea (Slavin, 1955). Later the intestinal stages of the parasite, the oocysts and the sporozoites were described in detail and differentiated from *C. baileyi*, the only other valid species infecting birds (Current *et al.*, 1986; Lindsay *et al.*, 1989). Oocysts are morphologically similar to *C. parvum, C. felis* and *C. wrairi* infecting the small intestines. This may contributed to misdiagnosis where identification is based solely on morphology and infection site. *Cryptosporidium meleagridis* occurs naturally in turkey but can experimentally infect chicken, rabbits, cattle or immunosuppressed mice (Sreter *et al.*, 2000). Similar strains of *C. meleagridis* have been reported in turkeys in the Czech Republic, Romania, Scotland, and US (Fayer, *et al.*, 2000).

The first documented human cases of *C. meleagridis* were in two patients with HIV, one residing in Kenya and the other in Switzerland (Morgan *et al.*, 2000a). The parasite was again identified in children in Peru where its occurrence was as high as that of *C. parvum* 'bovine' type in children who were not infected with HIV (Xiao *et al.*, 2001a). In this study, it appeared that *C. meleagridis* was part of the endemic enteric mix of organisms infecting the local population perhaps only being detected now with the current molecular techniques. In a molecular epidemiological analysis of *Cryptosporidium spp* in UK, in 1,705 faecal samples, *C. meleagridis* was identified in 0.3% of the samples in immunocompetent people (McLauchlin *et al.*, 2000). Morphology and molecular analysis based on the 18S rRNA gene suggests the species is closely related to the *C. parvum* group. The current data suggest *C. meleagridis* may be a common infection in humans with or without immunosuppression but is misidentified due to its similarity to *C. parvum.*

Further research on whether the parasite infects other hosts routinely will reveal whether the species can remain a distinct species on the basis of turkey being the known natural host (Morgan *et al.*, 1998). Human infections with *C. baileyi* seem more rare. A case of *C. baileyi* was described in a HIV infected person from Austria (Ditrich *et al.*, 1991). While extensive morphology, experimental infection and infection sites were carried out in this report, the lack of molecular typing data makes the diagnosis inconclusive.

5.8.2 *C. parvum* 'dog' type (*C. canis*) and *C. parvum* 'cat' type (*C. felis*)

Cryptosporidium parvum was first detected in a dog in USA by Wilson and others (1983). Thereafter most cases of *C. parvum* in dogs have been asymptomatic unless there is concurrent infection with canine distemper virus that is known to cause immunosuppression. Experimental infections in dogs with *C. parvum* from humans do not produce a patent infection although antibody production can be induced (Fayer *et al*., 1997). Current genotyping techniques have confirmed that the *C. parvum* 'dog' genotype is a distinct species from the two *C. parvum* commonly infecting humans (Fayer *et al*., 2001).

The first documented cases of human infection with *C. parvum* 'dog' genotype were in immunosuppressed patients (Pieniazek *et al*., 1999). In the same study, *C. 'felis*' was also identified in immunosuppressed patients. The authors proposed the recognition of the two as new distinct species although the morphometric details are lacking. A consequent report identified the presence of *C. felis* in six (27%) of the examined HIV-infected persons from USA and Switzerland (Morgan *et al*., 2000a). Among these patients, only three reported contacts with cats. These initial studies being the first ones to identify the unusual *Cryptosporidium* types in HIV patients suggested that immunosuppression may present a unique susceptibility to infections from different *Cryptosporidium* species and genotypes from the environment. However, while there may be an increased susceptibility, the parasites do not exclusively infect immunosuppressed humans. A study on *Cryptosporidium* parasites from patients in the UK identified four infection with *C. felis,* two of whom were immunocompetent while the other two had underlying severe illness, one being HIV

positive (Pedraza-Diaz *et al*., 2001a). One case of *C. parvum* 'dog' type was identified in the same study in an immunocompetent person. Another study in Peru identified one case of *C. felis* and two cases of *C. parvum* 'dog' type amidst cases of cryptosporidiosis mainly due to infections with *C. parvum* 'human' and 'bovine' type (Xiao *et al*., 2001a). These studies suggest that there may be more species involved in zoonotic cryptosporidiosis in both immunocompetent and immunocompromised persons than previously thought. Recently, *C. parvum* 'cervine' genotype has been isolated from humans in British Columbia (Ong *et al*., 2002). The authors suggested the source of infection to humans could have been water contamination with oocysts from wildlife.

The extent of susceptibility, the scope of potential pathogens and their transmission dynamics, whether they can re-infect animals from the human hosts is still unclear (Xiao *et al*., 2000a). Recent identification of *C. felis* in cattle (Bornay-Llinares *et al*., 1999) coupled with the reports in humans and natural feline hosts, goes to show further the complexity of transmission and susceptibility patterns of the parasites in this genus.

5.9 Diagnosis

There are numerous methods of detecting cryptosporidiosis either through immunological assays (serology or antigens in stool) or detection of oocysts by staining, immunofluorescence or molecular techniques (Weber *et al*., 1991; Arrowood, 1997). To detect oocysts, tissue biopsy or stool samples have been used successfully. Stool specimens need to be examined fresh or be well preserved either

in fixative such as 10% buffered formalin, sodium acetate formalin, or in storage medium such as aqueous potassium dichromate (2.5% w/v) (Fayer *et al.*, 1997). A recent report suggests preservation of oocysts in 75% ethanol at room temperature maintains oocysts morphology and they are suitable for molecular analysis (Jongwutiwes *et al.*, 2002). Oocyst shedding varies in all stages of infections therefore several stool samples need to be examined before giving a negative diagnosis.

5.9.1 Staining for detection of oocysts

Initial identifications of *Cryptosporidium* oocysts were by histological staining of infected gut tissue, mucosal scrapings, or gut contents of infected animals (Tyzzer, 1910; Slavin, 1955). Giemsa staining was later found to stain oocysts in stool although the method has low specificity (Tzipori *et al.*, 1980). Later, Henriksen and Pohlenz (1981), described the use of acid-fast Ziehl Neelsen staining, a method that is still used extensively in both clinical and research investigations as it is simple and effective. Since then there have been numerous modifications of the acid fast staining, some as cold or hot, while some have incorporated detergents such the Kinyoun acid fast stain (Garcia *et al.*, 1983; Ma and Soave, 1983).

Alternative methods including negative stains have also been developed such as the hot safranin-methylene blue stain, a test that has an advantage of staining other coccidia as well such as *Cyclospora* and *Isospora* (Baxby *et al.*, 1984; Fayer *et al.*, 1997). Fluorescent stains include auramine-phenol, auramine-rhodamine and auramine-carbol-fuchsin among others are used routinely (Moodley *et al.*, 1991; Tortora *et al.*, 1992). For experienced microscopists, iodine wet mount, phase contrast

or differential interference contrast (Nomarski) on fresh or concentrated stool can be used for screening followed by confirmation with a more sensitive assay (Arrowood, 1997).

5.9.2 Immuno-assays for detection of *Cryptosporidium*

Direct fluorescent antibody and ELISA are becoming increasingly more common in the detection of *Cryptosporidium*. These methods have the advantage of being more sensitive and less user-dependent compared to acid fast staining (Fayer *et al.*, 1997). Other methods include agglutination assays using latex beads coated with anti-sera from oocyst-immunized rabbits, which are then used on homogenised stool samples. The method however showed low specificity recording some false positives (Pohjola *et al*, 1986). Current commercial kits have shown sensitivities of 98-99% for *Cryptosporidium* (Garcia *et al.*, 2000). The MeriFluor™ (Meridian Diagnostics Inc.), a direct immunofluorescence assay for *Cryptosporidium* and *Giardia* has shown high sensitivities and specificities of between 96-100% compared to acid fast staining.

5.9.3 Serodiagnosis of cryptosporidiosis

Serologic diagnosis in cryptosporidiosis may be useful in transient disease where oocysts excretion is intermittent or has ceased. Diagnosis is based on demonstrating significantly high levels of anti-cryptosporidial antibody titres, or, identification of antigen-specific antibody responses to target antigens. The method has drawbacks for use in clinical diagnosis as detectable antibodies appear approximately two weeks post infection when the infection is resolving and persist long after recovery. It is

applicable in epidemiological surveys as high antibody titres persist for up to one year (1:40-1:640) (Campbell and Current, 1983).

5.9.4 Molecular Techniques for detection of *Cryptosporidium*

Molecular biologic techniques for the detection of *Cryptosporidium* have gained significance in the last decade (Fayer *et al.*, 2001). Detection of the organism in environmental samples using conventional techniques such as microscopy is problematic as oocysts may be few and extremely hard to detect. Molecular methods have had their biggest impact in differentiating species and strains through identification of characters applicable in classification of the organism.

5.9.4.1 Application

Early studies using karyotypic analysis of the chromosome sized DNA of *C. parvum* and *C. baileyi* showed the former had five chromosomal bands. These were estimated to be about 1500-3300 kilobases using inversion gel electrophoretic patterns. *C. baileyi* showed a distinct 6 chromosomal bands with a similar size range to *C. parvum* (Mead *et al.*, 1988). Later, more accurate studies showed *Cryptosporidium* has much lower chromosome size of 900-1400kb (Kim *et al.*, 1992), a conservative pattern compared to other protozoan parasites with highly variable chromosomal karyotypes such as *Plasmodium*, and *Leishmania spp* (Arrowood, 1997).

Other strain identification studies initially using pulsed field gel electrophoresis on a sporozoite protein, confirmed biological observations of the existence of geographically distinct, *C. parvum* strains. Some bovine isolates from Alabama and equine isolates from Louisiana were indistinguishable from each other, but were

distinct from bovine isolates from Iowa and human isolates from Peru and Mexico (Mead, *et al.,* 1990). The method was also able to distinguish three species (*C. parvum, C. baileyi* and *C. muris*) by analysing the oocyst wall protein. A significant application of molecular data derived from sequences from *Cryptosporidium* isolates has been on the phylogeny of the organism. Recently, several DNA and RNA regions of the *Cryptosporidium* organism have been identified and applied in phylogenetic studies especially in its relationship with other Sporozoan parasites (Xiao *et al.,* 1999a).

5.10 Phylogeny of *Cryptosporidium*

Molecular data especially based on DNA has become an important tool in the evolutionary studies. The use of morphological, behavioural, physiological and biochemical characters in taxonomy is largely impended by the effects of selection pressure that can lead to identical phenotypes of unrelated organisms developing under similar environments (Kunz, 2002). The vast majority of eukaryotic DNA sequences is non-coding (neutral DNA) and is not significantly controlled by selection. Since data are based on the DNA or protein molecule for all organisms, the data are more amenable to quantitative treatments than morphological data. Moreover, analysis can be applied directly to assess not only closely related organisms but also the links between ancient evolutionary occurrences such as the origin of mitochondria (Wen-Hsiung Li, 1997). The methods are also useful especially in microorganisms where limited number of morphological or physiological characters are available or give contradictory data for phylogeny.

The phylogenetic position of *Cryptosporidium* has been elusive although studies based on the analysis of the small-subunit (SSU) rRNA genes suggest that the genus constitutes an early emerging lineage from Apicomplexa (Zhu *et al.*, 2000a). Results from SSU rRNA indicate the genus does not form a monophyletic clade with other intestinal coccidia including *Eimeria, Isospora* or the cyst forming *Sarcocystis* or *Toxoplasma*. Instead the genus appears to be a sister-group to the Coccidia + Haematozoa (Escalante and Ayala, 1995; Barta *et al.,* 1997). These data show Coccidia is monophyletic only if the genus *Cryptosporidium* is excluded and could actually be used as an out-group for this class. Although studies suggest *Cryptosporidium* may be an early emerging lineage among the Apicomplexa, bootstrap support appears to be low suggesting the need for further research (Carreno *et al.* 1999; Zhu *et al.*, 2000a). It has however been proposed that perhaps this is as a result of the exclusive use of SSU rRNA for most phylogenetic analysis of the Apicomplexa which might inherently propagate some misinformation for specific organisms (Escalante and Ayala, 1995; Kim, 1996).

Recently, there have been other studies to re-evaluate this position. One study targeted the SSU rRNA, fused SSU/large-subunit (LSU) rRNA and six proteins namely, α- and β-tubulin, cyclin-dependent protein kinase (cdc2), dihydrofolate reductase-thymidylate synthase (DHFR-TS) cytosolic 70 kDa heat shock protein (HSP70), and elongation factor EF-1α (Zhu *et al.*, 2000a). The study compared 52 complete SSU rRNA sequences representing diverse genera within the phylum Apicomplexa and other closely related taxa. Species included were from Ciliophora, Dinozoa, Haematozoa, and Coccidia against different species of *Cryptosporidium* while species

from divergent genera such as *Caryospora, Hepatozoon* and *Perkinsus* were omitted, as only partial sequences were available. Analysis using maximum likelihood (ML), neighbour joining (NJ) and maximum parsimony (MP) phylogeny on the SSU rRNA gene showed support that *Cryptosporidium* genus is a sister-group of the Coccidia + Haematozoa (bootstrap value 73%) rather than a member of class Coccidea. The authors suggested it had an early emergence of the genus from the class Coccidea. The effect of long branch attraction artefact (LBA) was calculated using the S-F method (Brinkmann and Philippe, 1999) and the analysed data confirmed that the early emergence of the organism is real probably due to a few very slowly evolving positions within the SSU rRNA gene (Zhu *et al*, 2000a).

The study also evaluated the phylogenetic position of *Cryptosporidium* using the LSU rRNA sequences aligned with those from 8 other species including some Ciliophora, Dinozoa, Haematozoa and other Coccidia. Analysis by maximum likelihood, neighbour joining and maximum parsimony all resulted in trees congruent with the current taxonomy of the Apicomplexa (Zhu *et al.*, 2000a). The results show the Coccidia are monophyletic but *Cryptosporidium* emerged in two significantly different phylogenetic positions. In the maximum likelihood and the maximum parsimony, it emerged at the base of the Apicomplexa (bootstrap 99%) while in the neighbour joining, as a sister-group to *Plasmodium* (bootstrap 60%). This discrepancy was attributed to different sensitivities of the methods used in tree construction or model violations (Kim, 1996; Zhu *et al*, 2000a). Phylogenetic reconstruction using six proteins favoured the early emergence of *Cryptosporidium* but only with moderate support (bootstrap 45-64%). Within the protein analysis, β-tubulin and DHFR-TS

placed the organism at the base of the Apicomplexa (bootstrap 70-85%) in agreement with the position based on the SSU rRNA gene. However, in the α-tubulin and HSP70 analysis, *Cryptosporidium* emerged as a sister-group to *Plasmodium* (bootstrap 79% and 64% respectively). The study concluded that all the methods SSU rRNA, LSU/SSU rRNA, and the six proteins supported the early emergence of *Cryptosporidium*. A phylogenetic maximum parsimony tree based on 18S rRNA gene from the Apicomplexa is shown below (Fig. 5.2). In this model, *Perkinsus marinus* was used as an outgroup, and *Cryptosporidium* emerged as a sister group to the gregarines rather than among the other coccidian.

The low support for some of the phylogeny constructions and discrepancies observed suggest further research is necessary to clarify the phylogenetic position of the organism. The results proved that the observed differences were not a result of LBA artefacts. Still the data could not categorically determine the position of *Cryptosporidium* since there may be other limitations in the reconstruction models. Conversely, there may be genuine differences in the genome and the biology of the genus from its closest relatives that set it apart (Coombs, 1999). So far, biochemical, drug testing, ultra-structural data and now molecular phylogenetic data are consistent with the emergence of *Cryptosporidium* at the base of the Apicomplexa (Woods *et al*., 1996; Coombs, 1999; Riordan *et al*., 1999; Zhu *et al*., 2000b).

Fig 5.2 Phylogram of Apicomplexan parasites based on the 18S rRNA gene

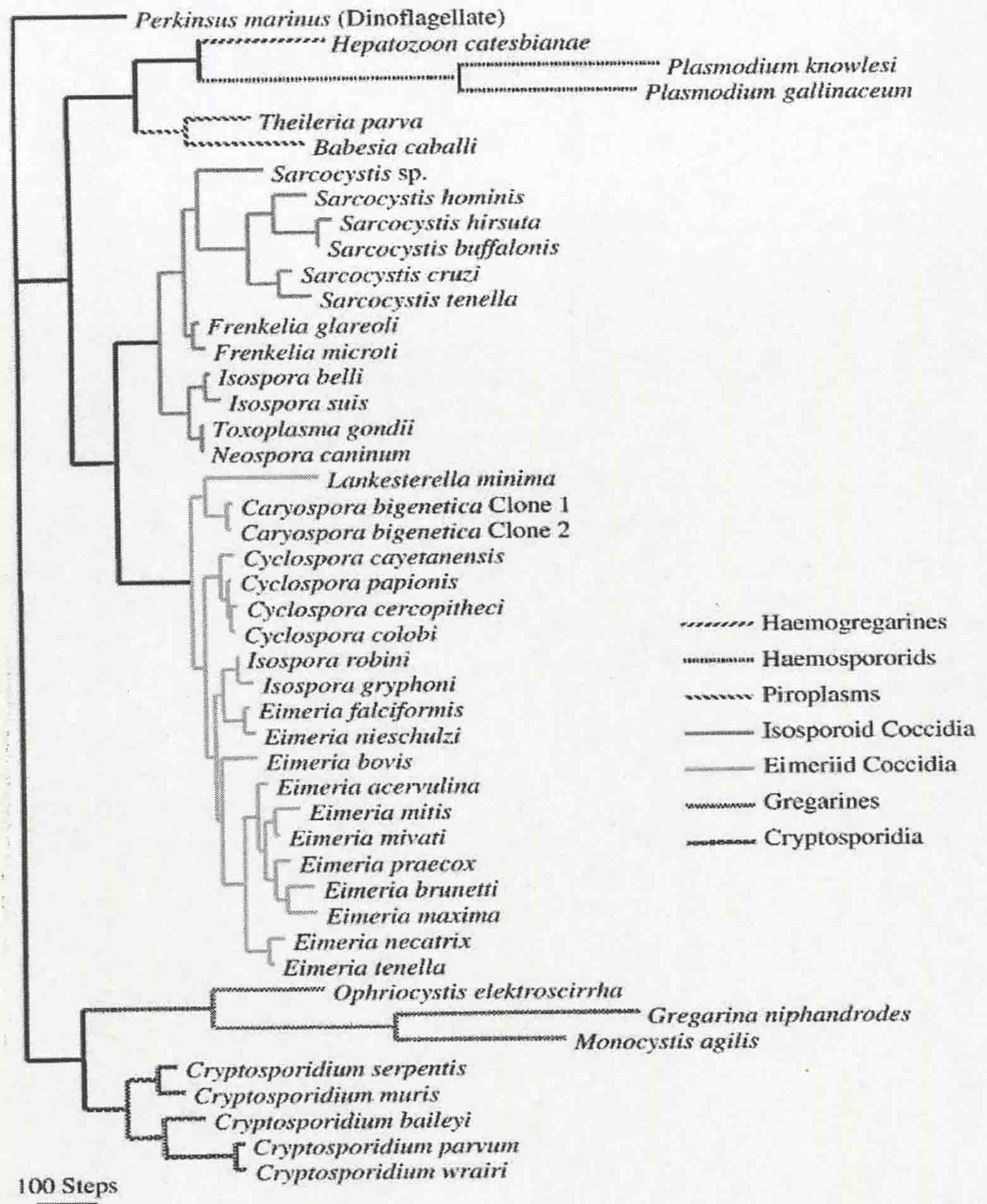


Fig 5.2 shows genetic relatedness of representative samples of apicomplexan parasites based on 18S rRNA gene using a Maximum parsimony phylogram *Perkinsus marinus* was used as theout group. Note the emergence of *Cryptosporidium* as a sister group of the gregarines rather than a member of the coccidea. (Figure adapted from Barta *et al.*, 2001).

Recent information from *in vitro* studies suggests the organism is closer to the gregarines (Hijjawi *et al.*, 2001). Another study of the SSU rRNA has also shown that the gregarines form a separate monophyletic clade, are a sister group to *Cryptosporidium* and the two taxa emerge early from the Apicomplexa (Carreno *et al.*, 1999). Current limitations in elucidating some of the uncertainties is that there are still too few sequences of diverse species available in the database to determine phylogeny based on specific genes such as the LSU rRNA or protein sequence. Extensive research both in the organism and other related groups (such as Dinozoa and Ciliophora) will determine whether *Cryptosporidium* is truly ancestral to the Apicomplexa or if its features are by 'acquisition' following loss of genes, organelles and/or functions (Zhu *et al.*, 2000a).

5.11 Genotype analysis of *Cryptosporidium spp*

The *Cryptosporidium* genome is comprised of 10.4 MB in eight chromosomes ranging from 1.04 to 1.5Mb. Since the genome encodes all the heritable information that determines the biological characteristics of the parasite its understanding provides necessary information required for target research into epidemiology, prevention and treatment of the resulting disease (Liu *et al.*, 1999).

Lately, genetic markers for *Cryptosporidium* species have been identified (Awad-El-Kariem *et al.*, 1994; Morgan *et al.*, 1995; Morgan *et al.*, 1999b; Xiao *et al.*, 1999a; Xiao *et al.*, 1999b). Before then the extent of genetic polymorphism in *Cryptosporidium* organisms infecting humans and other mammals had not been fully appreciated. Experimental animal infections suggested there are at least two

phenotypically different strains with one appearing to be zoonotic while the other was anthroponotic (Dubey *et al.*, 1990). Genetic polymorphism was first used by Ortega and others, (1991) using RFLP and non-specific human DNA probes. They examined genetic differences that confirmed the phenotypic characteristics showing humans harboured two different isolates, one from calves and the other solely a human-to-human type. Isolates from humans were similar from different geographical regions using random amplification of DNA (RAPD) or RFLP (Carraway, *et al.*, 1996; Tilley and Upton, 1997).

Identification of a 2.8kb threonine-rich open reading frame from *C. parvum* further allowed genetic identification and characterisation of two distinct *C. parvum* genotypes, again corresponding to the two transmission pathways (Tilley and Upton, 1997; Spano *et al.*, 1998). Application of restriction endonucleases resulted in smaller fragments that could be sequenced easily for confirmation of genetic polymorphism of the organisms (Morgan *et al.*, 1995; Peng *et al.*, 1997; Morgan *et al.*, 1998; Sulaiman *et al.*, 1998; Xiao *et al.*, 1999b). In a multilocus study on polymorphism, Spano and others (1998), analysed five polymorphic loci from 28 isolates from Europe, North and South America, and Australia. The results of the five gene targets showed all isolates of *C. parvum* clustering into these two distinct groups. The genes studied included *Cryptosporidium* oocysts wall protein locus (COWP), polythreonine (polyT) locus, and the gene encoding thrombospondin-related adhesive protein of *Cryptosporidium* (TRAP-C1). They demonstrated the occurrence of two separate alleles each associated with either human or bovine isolates. The other genes were ribonucleotide reductase (RNR) while the internal transcribed spacer (ITS) region

was used as the genus specific marker. In each locus, two electrophoretic profiles were observed with no re-assorted genotypes indicating two distinct genotypes. The absence of recombinant genotypes suggested the two have evolved separate reproductive cycles in spite of them occurring at times, in the same host.

Improvements on genotyping techniques, coupled with sequencing and phylogenetic analysis illustrate the diversity of *Cryptosporidium* organisms and polymorphism in specific genes. This has led to suggestions by some researchers that the two genotypes of *C. parvum* are separate species implying there is a need for revision of taxonomy of the genus *Cryptosporidium* (Morgan *et al.*, 1995; Morgan *et al.*, 1999d; Xiao *et al.*, 1999a; Pieniazek *et al.*, 1999; Morgan *et al*, 2000b).

5.11.1 Target genes for identification of *Cryptosporidium*

Genomic DNA sequences have been extensively used for phylogenetic studies with the rRNA gene a most common target in both prokaryotes and eukaryotes (Woese and Fox, 1977). While many genes are targeted for the diagnosis of *Cryptosporidium*, the 18S rRNA gene and the HSP70 gene fragments remain the most reliable when primers designed from the *C. parvum* sequence are used (Xiao *et al.*, 1999b). PCR on COWP gene, TRAP-C1 and TRAP-C2 gene fragments failed to amplify samples positive with non-parvum *Cryptosporidium* organisms that were later shown to be *C. felis* and *C. canis* with the use of 18S rRNA gene and HSP70 gene fragments (Pedraza-Diaz *et al.,* 2001a).

In *Cryptosporidium*, the small subunit ribosome like in other eukaryotes has one ribosomal RNA molecule consisting of the 18S rRNA gene of approximately 2000 nucleotides. The 18S rRNA gene varies according to the species and genotype of *Cryptosporidium*. Overall, the *C. parvum* and related species have gene sequences of approximately 1733-1750bp with *C. parvum* 'human' genotype being the longest while *C. baileyi,* has the shortest. Most of the intraspecies differences occur in the first half of the gene with *C. parvum* showing the greatest subtype variation. The other group that includes *C. muris, C. serpentis* have slightly longer 18S rRNA gene sequence of approximately 2100bp. Early studies of this gene had brought more confusion than clarification, first indicating a 99% identity between *C. parvum* and *C. muris* (Cai *et al.*, 1992) but this was later found to have been due to misidentification and sequence inaccuracy.

Analysis of the complete gene has shown the polymorphism tallies with the biological differences in the *Cryptosporidium* genus and that the parasite is not closely related to other coccidia at this locus (Xiao *et al.*, 1999a). Subsequent studies show the gene has reliable sequences that are not only genus specific, but demonstrate the extent of intragenotypic variation that can aid in classification (Morgan *et al.*, 1998; Morgan *et al.*, 2000d; Morgan *et al.*, 1999c; Xiao *et al.*, 1999b). Sequence variation in the gene show the *Cryptosporidium* has two distinct groups according to their predilection site namely those primarily infecting the intestines or respiratory tracts (*C. parvum, C. baileyi, C. meleagridis*) and those infecting the stomach (*C. muris* and *C. serpentis*). The differences between the two groups are greater than the differences between *Eimeria tenella* and *Cyclospora cayetanensis* (Xiao *et al.*, 1999a).

The 'human' and 'bovine' genotypes of *C. parvum* differ in four regions of the gene while the latter and *C. wrairi* also differ in another four regions. The differences between *C. parvum* group and *C. wrairi* (isolate infecting Guinea pigs) at this gene level are not major and suggest the latter may be a subspecies of the former. The locus demonstrates the intraspecies variation within *Cryptosporidium spp* yet maintaining significant diversity within the genus making it a reliable diagnostic target (Xiao *et al.*, 1999a; Xiao *et al.*, 1999b). Like *Plasmodium*, the rRNA genes occur in low copy numbers and are distributed in single copies at different chromosomes (Goman *et al.*, 1991; Le Blancq *et al.*, 1997). However, unlike other eukaryotes, *Plasmodium* genus show developmentally controlled distinct SSU rRNA genes that include sporozoite stage-specific and blood stage-specific genes that contribute further, to the heterogeneity in the organism at this locus (Qari *et al.*, 1994; Li *et al.*, 1997). So far the differences between the heterogeneous copies in *Cryptosporidium* are small and their effects can be minimized with the use of appropriate models in phylogeny (Xiao *et al.*, 1999c).

6.0 CHAPTER 6: DETECTION AND IDENTIFICATION OF *CRYPTOSPORIDIUM* ISOLATES

6.1 Introduction

This section describes the materials and methods, results and discussion of acid fast staining, PCR, and genotype analysis of *Cryptosporidium* organisms identified in the study based on the 18S rRNA gene fragment in relation to geographical origin of sample and HIV status of the patients.

6.2 Materials and Methods

6.2.1 Sample sources

A total of 356 samples from the Kisumu/Busia cohort collected through the three visits from both mothers and children, were examined for the presence of *Cryptosporidium* organism. These were samples that were preserved in 2.5% potassium dichromate and could therefore be processed for PCR. *Cryptosporidium* was identified in 25/356 samples. Another 27 samples positive with *Cryptosporidium* were collected from the diagnostic laboratory of the Center for Microbiology Research, KEMRI.

Other isolates confirmed positive with *Cryptosporidium* by microscopy, were obtained from the Department of Medical Microbiology and Genito-Urinary Department, University of Liverpool, as shown in Table 6.1 below. Every sample contained patient's identification number, if child or adult, HIV status, and country of origin as shown in Table 6.1 below. Purified *Cryptosporidium* DNA (positive

controls) were kindly provided by Dr Lihua Xiao (Centers for Disease Control and Prevention, Chamblee, Atlanta, Georgia, USA).

Table 6.1 Number and Source of Samples used in the study

Sample Source	Isolates Number	Patients	Preservation	Status
Kisumu/Busia	15	**Adults**	2.5% P. dichromate	HIV +/HIV -
(Kenya)	10	**Children**	2.5% P. dichromate	HIV +/HIV -
CMR (KEMRI)	27	**Adults**	2.5% P. dichromate	HIV+/HIV-
Samples from other regions				
Malawi	12	**Children**	Frozen (-80^{0}C)	HIV-/HIV+
Thailand	36	**Adults**	Frozen (-80^{0}C)	HIV+
Vietnam	3	**Adults**	Cool (4^{0}C)	HIV+
Brazil	7	**Children**	Frozen (-80^{0}C)	HIV-
UK/Ireland	12*	**Children**	Cool (4^{0}C)	Untested

*1 isolate was from a captive monkey in a UK zoo.

All samples obtained from Kisumu/Busia cohort were examined by acid fast staining using the modified Ziehl Neelsen method as described in Appendix 4. In all others, *Cryptosporidium* oocysts had been confirmed elsewhere using acid fast staining and IFA test.

6.2.2 *Cryptosporidium* genomic DNA Extraction and PCR

Faecal samples were stored either fresh/frozen at –80^{0}C, or preserved in 2.5% potassium dichromate. Where samples were preserved, all potassium dichromate was washed off thoroughly by rinsing out with distilled/deionised water 5-6 times until the

yellow colour cleared. Frozen stool was aliquoted by scraping into a clean Eppendorf and suspended in lysis buffer (without thawing the stock sample).

6.2.2.1 Oocysts rupture

Reagents:

Extraction (Lysis) Buffer:	(50ml total volume)
0.5M EDTA	1.0ml (10mM)
5M NaCl	1.2ml (120 mM)
1M Tris ph7.5	1.25 ml (25mM)
Sarkosyl detergent	1ml (1%)
HPLC Water	45.55ml

Procedure

1. A pea size stool sample was scraped from frozen stool into 1.5 ml Eppendorf tube and 300μl of lysis buffer added. For samples preserved in potassium dichromate, the pellet from the final wash was re-suspended in 300μl of lysis buffer.
2. The sample was vortexed continuously for 30 seconds until completely emulsified. Oocysts were ruptured by subjecting them to five freeze-thaw cycles (freezing at -80^{0}C for 30 min and thawing in a water bath at 80^{0}C for 10 min) in extraction buffer as described by Kim *et al.,* (1992).

6.2.2.2 Proteinase K digestion

After the final thaw, samples were allowed to cool, then 40 µl of 10µg/ml proteinase K was added and mixed by vortexing. The mixture was incubated at 56^0C for 1 hour and agitated every 15 minutes.

6.3 DNA Extraction:

6.3.1 Protocol 1.

For the first 12 samples from Kisumu/Busia cohorts, DNA was extracted using the phenol/chloroform method (Protocol 1) as follows:

Reagents:

Tris buffered phenol (Sigma-Aldrich Co Ltd, UK)

Phenol:Chloroform: Isoamyl alcohol (Sigma-Aldrich Co Ltd, UK) at 25:24:1 ratio

Chloroform: Isoamyl alcohol at 24:1 ratio

1M Sodium acetate

100% cold ethanol

Procedure

DNA extraction

1. After the proteinase-K digestion (at 56^0C), the sample was allowed to cool to room temperature.
2. 1 total sample volume (tsv) of approximately 520µl (sample and lysis buffer suspension) of cold Tris-buffered phenol was added.
3. The mixture was then vortexed briefly (10 sec) and centrifuged at 13,000 revolutions per minute (rpm) for 10 minutes in a bench microfuge, at room temperature.

4. The aqueous supernatant was recovered into a clean 1.5ml Eppendorf tube.
5. Into the supernatant 500μl of phenol: chloroform: Iso-amyl alcohol was added.
6. The mixture was again vortexed for 10sec and centrifuged at 13,000 rpm in a microfuge for 10 minutes at room temperature.
7. The aqueous phase was recovered into a clean Eppendorf tube and into the supernatant, 1 tsv (equal to total supernatant recovered) of chloroform:Isoamyl alcohol was added, mixed and centrifuged for 10 minutes at 13,000 rpm.
8. The aqueous phase was recovered into a clean tube and the tsv (approximately 400 μl) recovered for DNA precipitation in high salts.

6.3.3.1 DNA precipitation

Reagents

1M Sodium acetate

100% ethanol

80% ethanol

HPLC water

Procedure

1. Into the approximately 400μl sample recovered above, 10% (40μl) of 1M Sodium acetate was added.
2. Into the mixture, 2.5 tsv (approximately 1ml) of cold absolute (100%) ethanol was added to precipitate the DNA and the solution mixed gently by inversion of the tube.
3. The sample was stored at -20^{0}C overnight to allow precipitation of the DNA.
4. The sample was removed from -20^{0}C and centrifuged at 13,000 rpm in a microfuge for 30 min at 4^{0}C.

5. The alcohol was removed and the pellet washed with 80% ethanol by adding an equivalent of 2X (approximately 800μl) volume of the original tsv, and centrifuged for 30 min at 4^0C.
6. The ethanol was removed and the DNA pellet allowed dried by leaving the tube open at room temperature for approximately 1-2 hrs.
7. The DNA pellet was re-suspended in 40ul of HPLC water and stored at -20^0C until use.

6.3.4 Protocol 2.

For all the other samples from Kisumu /Busia cohorts, Malawi, Thailand, Vietnam, Brazil, UK/Ireland and Vietnam, DNA was extracted using a commercial kit. The kit used was the QIAamp DNA extraction kit for Stool DNA (QIAGEN Ltd, Crawley, West Sussex, UK) according to the manufacturers instructions. The kit was stored at room temperature until expiry date as indicated. One kit contained reagents enough for 50 extractions.

6.3.4.1 Oocysts rupture and DNA extraction

The Qiagen kit is designed for DNA extraction from enteric pathogens in stool that can be lysed easily including bacteria and viruses. However, it is also applicable for extraction of pathogen DNA in those with oocyst wall or spores with modification or prior rupture of oocysts.

6.3.4.2 Summary of the Qiagen kit procedure

All reagents were provided in the kit with the exception of 100% ethanol.

Organic products of pathogens present in the stools were recovered by lysis into the buffer. Large faecal debris was then precipitated by centrifugation and the supernatant containing proteins and nucleic acids recovered. Excess impurities were then removed from the supernatant by the application of a tablet (provided in the kit) that dissolves excess organic materials and removes other PCR inhibitors. Proteins were digested with the use of proteinase K enzyme also included in the kit. Digested proteins and excess enzymes are then washed off with buffers while the DNA is bound in the presence of high salts onto the membranes in the tubes provided. DNA was then eluted from the membrane with HPLC water into a collecting tube and stored at –20^{0}C until further use.

6.3.4.3 Modification of DNA extraction procedure

To enhance the success of DNA extraction from oocysts of *Cryptosporidium* in stool samples, lysis buffer (*ASL* Buffer-provided) was added and used to lyse pathogens during the freeze/thaw process described above. (The instructions in the kit state the lysis buffer should be added followed by 10 min incubation at 70^{0}C for 10 minutes and DNA extraction). Approximately 250µl of *ASL* lysis buffer was used to suspend the faecal aliquot scrapings from frozen samples. The mixture was vortexed until completely homogenised. Samples were then subjected to a five, freeze-thaw cycle as described above. After the final freeze-thaw, an additional 1.2 ml *ASL* lysis buffer was added into the stool mixture and the extraction followed the procedure described according to the manufacturer's instructions.

6.4 PCR of *Cryptosporidium* DNA

Principle

PCR targeted part of the 18S rRNA gene of the *Cryptosporidium* organism in the isolates processed. The region is highly polymorphic within the *Cryptosporidium* genus but with little similarity to other sporozoan parasites. Nested PCR where a larger fragment of approximately 1325bp is first amplified followed by second amplification of an internal fragment (approximately 840bp) of the primary product was used to increase sensitivity of the test. The usefulness of the method in identification of *Cryptosporidium* and subsequent genotyping of the organism has been demonstrated (Xiao *et al.,* 1999b).

Procedure

Reagents

10X PCR buffer	(Perkin Elmer (PE) Applied Biosystems, UK)
2.5 mM dNTP	(PE Applied Biosystems, UK)
25mM Mg Cl_2	(PE Applied Biosystems, UK)
Ampli-Taq DNA Polymerase	(PE Applied Biosystems, UK)
HPLC Water	(BDH)

Table 6.2 Primer sequences and positions on the complete 18S rRNA gene

Primers	Position (*C. parvum* 18S gene)	Sequence
External		
AL 1687	156-175	5'-TTCTAGAGCTAATACATGCG-3'
AL 1691	1455-1475	5'-CCCTAATCCTTCGAAACAGGA-3'
Internal		
AL 1598	193-218	5'-GGAAGGGTTGTATTTATTAGATAAAG-3'
AL 3032	1008-1029	5'-AAGGAGTAAGGAACAACCTCCA-3'

Primers adopted from Xiao *et al.,* 1999b.

Primer position based on *C. parvum* (Accession number in GenBank AF09349) strain HCNV4 complete 18S rRNA gene. Primers synthesized by Genosys Oligonucleotides, (SIGMA Genosys Ltd Pampisford, Cambridgeshire, UK).

6.4.1 PCR mixture

Primary PCR master mix

Reagents	**100 μl Reaction volume**
10 x Perkin Elmer buffer	10μl
2.5 mM dNTP	16μl (200μM each)
25 mM $MgCl_2$	24μl (6mM final)
AmpliTaq DNA Polymerase	1.0μl (2.5U)
AL 1687	5.0μl (100nM)
AL 1691	5.0μl (100 nM)
DNA test sample	0.5-1.0μl (about 10ng-1μg DNA)
HPLC water	Top to final volume 100μl

6.4.2 Amplification

PCR cycling conditions

Initial denaturation	94^{0}C for 3 min
35 cycles	94^{0}C for 45s,
	55^{0}C for 45s
	72^{0}C for 1 min
Final extension at	72^{0}C for 7 min

The first round of PCR consisted of the above primary mixture and amplification conditions described. Reaction was in 200 μl PCR Eppendorf tubes in Techne (FTGENE2D Techne (Cambridge) Ltd., UK) thermal cycler. Samples were thoroughly mixed by vortexing, and then centrifuged for 30 seconds prior to loading onto the thermo-cycler. To minimize contamination, only designated pipettes and

barrier tips were used in the preparation of the PCR mixtures. Different working areas were used for DNA preparation and PCR procedures. The master-mix was prepared in the clean laminar flow cabinet as were the reaction aliquots. Disposable gloves were used and changed regularly for all procedures.

A negative control sample containing no DNA template was included in all PCR reactions and was negative each time.

Secondary PCR master mix

10 x Perkin Elmer buffer	10μl	
2.5 mM dNTP	16μl	(200μM each)
25 mM $MgCl_2$	12μl	(3Mm final concentration)
AmpliTaq DNA Polymerase	1.0μl	(2.5U each)
AL 1598	5.0μl	(200nM each)
AL 3032	5.0μl	(200nM each)
Primary PCR product	2.0μl	(0.5-1.0μl each)
HPLC water	Top volume to 100μl	

PCR mixture for the secondary reaction and amplification conditions were similar as in the primary PCR except the change in the final concentration of MgCl (3mM) that was half, the one used in primary PCR. The DNA template was the primary PCR product from the first reaction. Preparation and cycling conditions were similar to the primary PCR.

6.4.5 Agarose Gel Electrophoresis

Reagents and equipments

Agarose Ultra-Pure (Agarose, Life Technologies, Paisley, Scotland).

Ethidium bromide (Sigma Chemicals Co, St Lois MO)

1 Kb or 123 bp DNA ladders (GibcoBRL Life Technologies, UK)

Electrophoresis tank (Horizon 11.14 GibcoBRL, Life Technologies, UK)

Gel Documentation software (BioRad, UK)

TBE Buffer (5XTBE Buffer 54gms Tris base, 27.5 gms Orthoboric acid, 20mls 0.5MEDTA pH8.0)

Procedure

The presence and size of the PCR product were determined by agarose gel electrophoresis. A 2% agarose gel was prepared by adding 100ml of 0.5X Tris-borate-EDTA (TBE) buffer to 2g of agarose (The mixture was placed in a microwave oven until melted (approximately 3 minutes). The agarose was allowed to cool and 6 ul (0.05% w/v) of ethidium bromide was added, mixed and poured in a tray with a pre-positioned comb and allowed to set. The tray was then placed in a submarine electrophoresis tank and covered with additional 0.5 x TBE buffer to cover the surface of the gel. 5μl of secondary PCR products were then mixed with 2μl loading buffer (Bromophenol blue 0.05% w/v) and loaded onto the wells. A positive control sample of known purified DNA (from Division of Parasitic and Infectious Disease Unit, CDC, Atlanta) and a negative control sample were run along the test samples. A potential difference of 120volts was maintained across the tank for 1 hour, or until the

dye reached the edge of the gel on the positive anode. DNA bands were visualized by ultra-violet trans-illumination, and pictures were taken for records using a gel documentation system. PCR product was sized according to the migration of the DNA ladder. Positive control samples yielded an approximately 840-860bp PCR fragment.

6.5 Restriction Fragment Length Polymorphism

Principle

Restriction fragment length digestion using two different enzymes has been shown to differentiate most *Cryptosporidium* species. *Ssp*I and *Vsp*I endonuclease digestions differentiate *Cryptosporidium* species and genotypes of *C. parvum* and other closely related species such as *C. meleagridis, C. wrairi* and *C. parvum* 'monkey' genotype. Most non-parvum *Cryptosporidium spp* have a different restriction pattern with the first enzyme (*Ssp*I). However, in the case of the occurrence of unusual species it is necessary to do the restriction endonuclease coupled with sequencing to confirm identification.

Enzymes

Table 6.3 Restriction endonucleases source and cleavage positions

Enzyme	Biological Source	Restriction site
*Ssp*I	*Sphaerotilus spp*	AAT ↓ATT TTA ↑TAA
*Vsp*I	*Arthrobacter spp*	AT↓ TAAT TAAT↑TA

Enzyme Source: Boehringer Mannheim, UK

6.5.1 Restriction digestion reactions

<u>Reaction 1</u>

*Ssp*I (20U)	2.0µl
Buffer	5.0µl
Water	23µl
PCR product	20µl
Total Volume	50µl

<u>Reaction 2</u>

*Vsp*I (20U)	2.0µl
Buffer	5.0µl
Water	23µl
PCR product	20µl
Total Volume	**50µl**

Procedure

1. Restriction digests were incubated at at 37^{0}C for 1 hr.
2. The products were separated on 2% agarose gel electrophoresis as described above. The fragments were visualized by ultraviolet trans-illumination. Fragment sizes were then compared with the expected patterns from known *Cryptosporidium* species and genotypes.

Table 6.4 Predicted RFLP digestion patterns for *Cryptosporidium*

Species	Genotype	PCR product length (bp)	Visible Fragments (bp) *Ssp*I	*Vsp*I
C. parvum	Human (1) type	837	449, 254, 111	526, 104, 102,70
C. parvum	Bovine (2) type	834	449, 254, 108,	628, 104
C. parvum	Dog	829	417, 254, 105	633, 102
C. parvum	Monkey	835	461, 254 109, 11,	559, 104
C meleagridis	Turkey	833	449, 254, 108	456, 171,104
C. muris	Hyrax, camel	833	448, 385	731, 102
C. felis	Cat	864	426, 390, 33, 15	476, 182 104

Source: Xiao *et al.,* 1999b. Note only sizes of visible fragments are shown

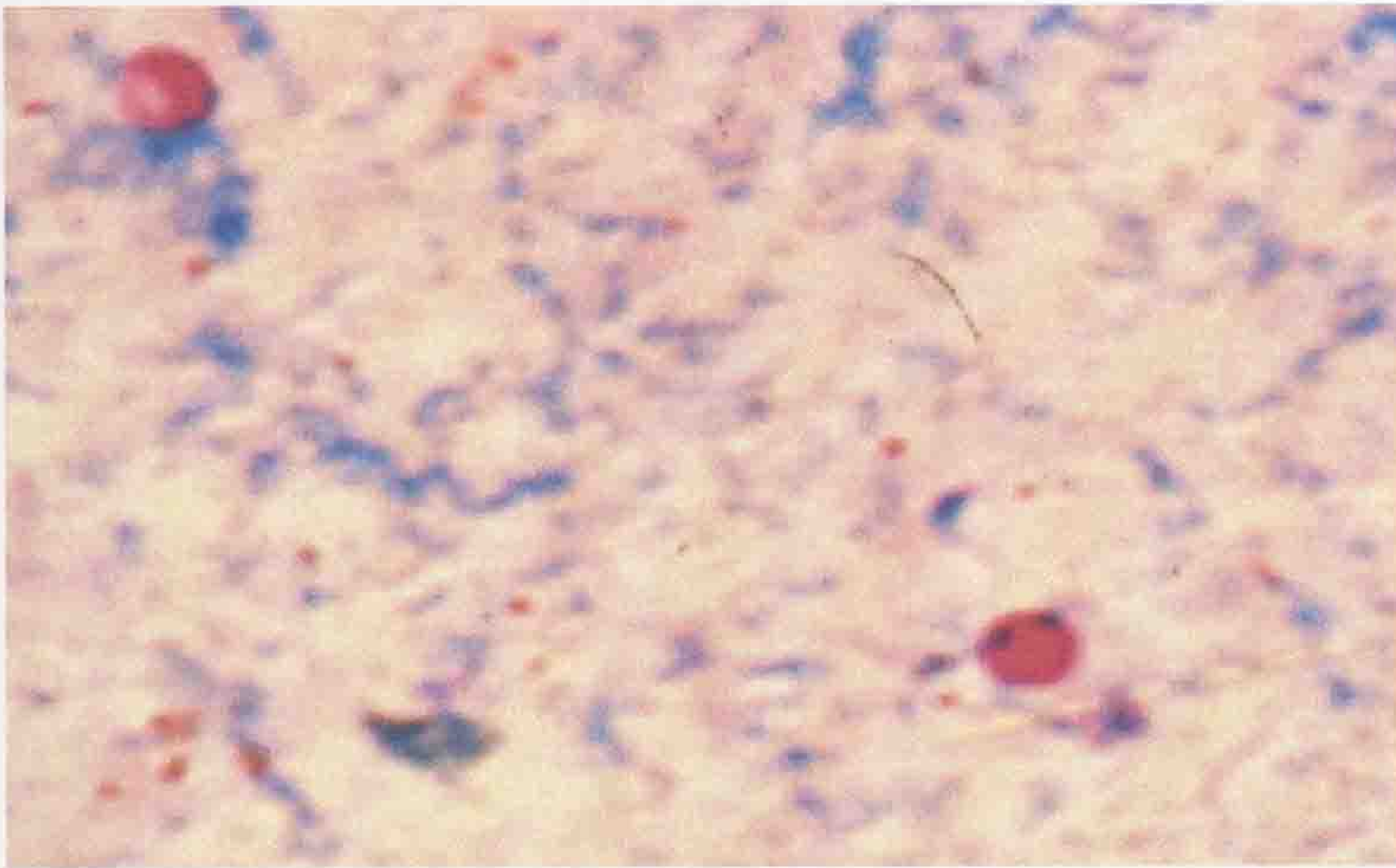
6.6 RESULTS

6.6.1 ZN acid fast staining

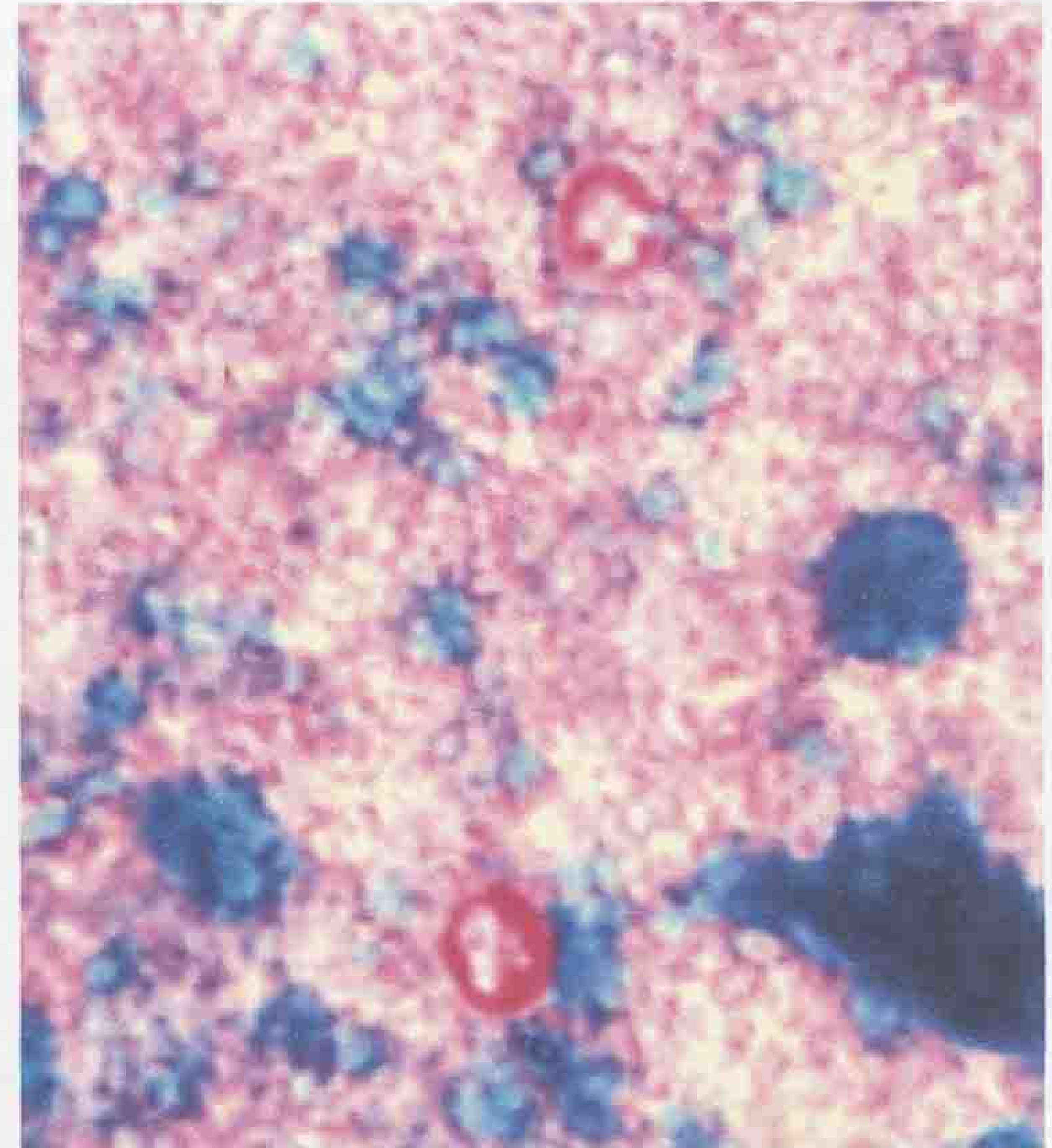
Samples from western Kenya cohort and those from Centre for Microbiology Research were stained by ZN acid fast and examined under oil immersion. A 7% (25/356) prevalence of *Cryptosporidium* was recorded from patient samples collected in Kenya cohort. This included 9/117 (7.7%) from HIV infected mothers and 3/19 (15.8%) from HIV infected children. Among HIV un-infected group, 6/139 (4.3%) were from mothers and 7/81 (8.6%) were from children. Acid fast staining and microscopy identified positive samples as having *C. parvum* or *C. parvum*-like oocysts. These oocysts appeared as ring-shaped pink to red bodies, approximately 4-5μm in diameter (Fig. 6.1a). One sample however, had oocysts that appeared larger than usual and were more ovoid than spherical, measuring an average size of 7.5-9.8μm x 5.5-7.0μm (Fig 6.1b) The oocysts were presumed to be *C. muris*. Three samples (3/356) showed the presence of *Isospora belli* (Fig 6.4c) including the sample with *C. muris*-like oocysts. All other samples from CMR (Kenya), Malawi, Brazil, UK, Vietnam and Thailand were already confirmed as *Cryptosporidium* positive by microscopy and IFAT.

Fig 6.1 Coccidian parasites identified in the study

a)



b)



c)

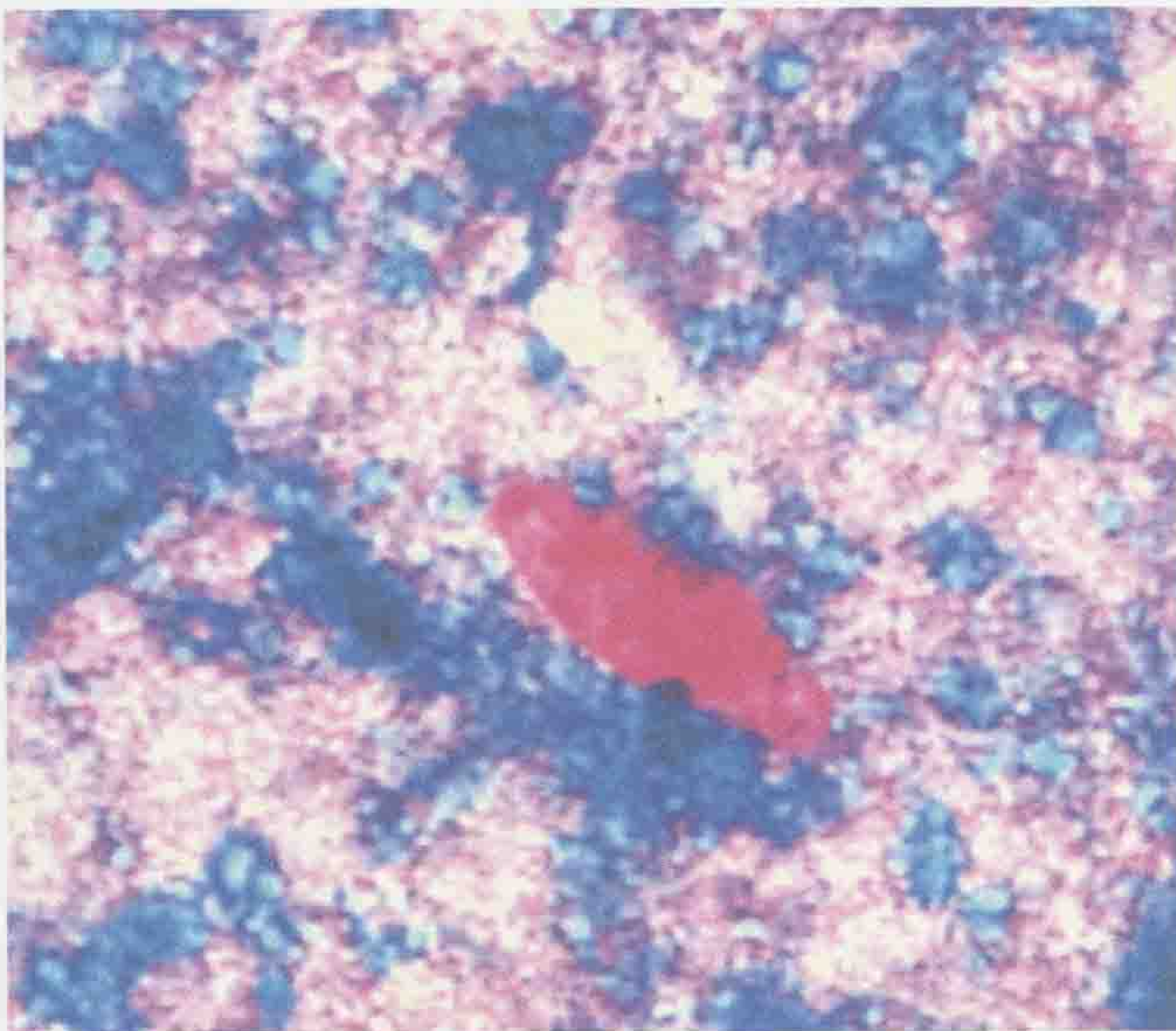


Fig 6.1 shows the sizes of the different oocysts identified

		Typical Size
a)	*C. parvum*	4-6μm
b)	*C. muris*	7.5-8.5μm
c)	*Isospora belli*	25X 19μm

6.6.2 *Cryptosporidium* DNA Extraction.

All samples processed for DNA extraction and PCR were presumed positive for *Cryptosporidium* based on the acid fast staining. Of the 12 samples initially extracted using phenol:chloroform:isoamyl method, only 5 amplified in the consequent PCR reactions. This method of extraction was therefore discontinued and a commercial kit used to recover DNA from all the remaining stool samples. Still, isolates preserved in potassium dichromate had lower efficacy in consequent PCR reactions. Of the remaining 40 samples preserved in potassium dichromate, 31 (78%) resulted to successful amplifications and a visible product. This was however, lower compared to the successful PCR amplifications on samples that were fresh frozen (at -80^0C) which showed a 94% success rate.

6.6.3 Nested PCR

Primary PCR yielded a product of approximately 1325 bp in length. A secondary PCR, targeting an internal fragment of the primary product yielded a single product that corresponded to the expected size of between 829-864bp.

Fig 6.2a Results of Nested PCR for *Cryptosporidium spp*

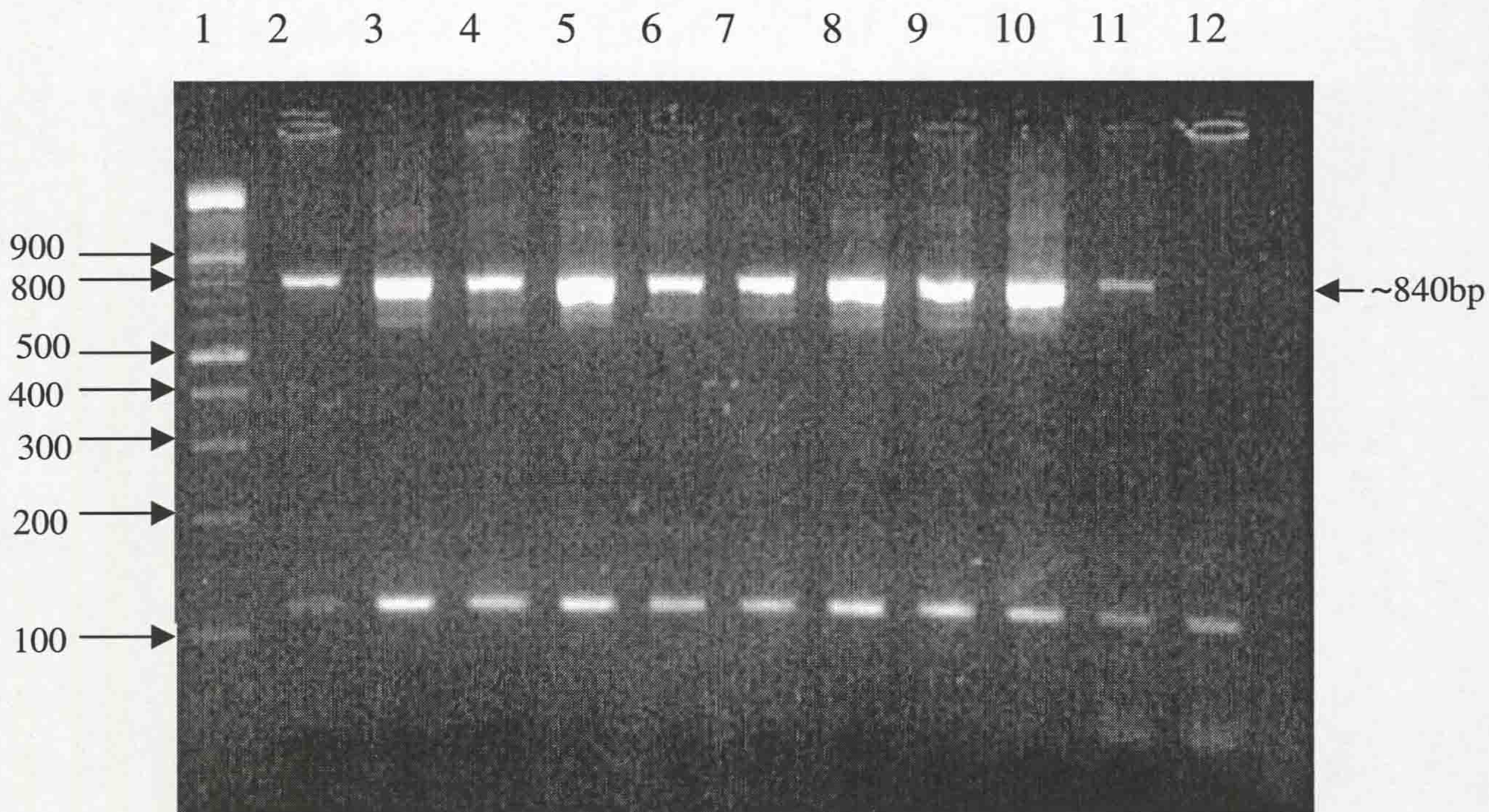


Fig 6.2a Visualisation of PCR product generated after secondary PCR. Lanes 1 shows the 100bp molecular weight marker. Lanes 2 shows the positive control while Lanes 3-11 show the samples from the study. Lanes 12 was the negative control sample.

Fig 6.2b PCR on selected samples from Thailand

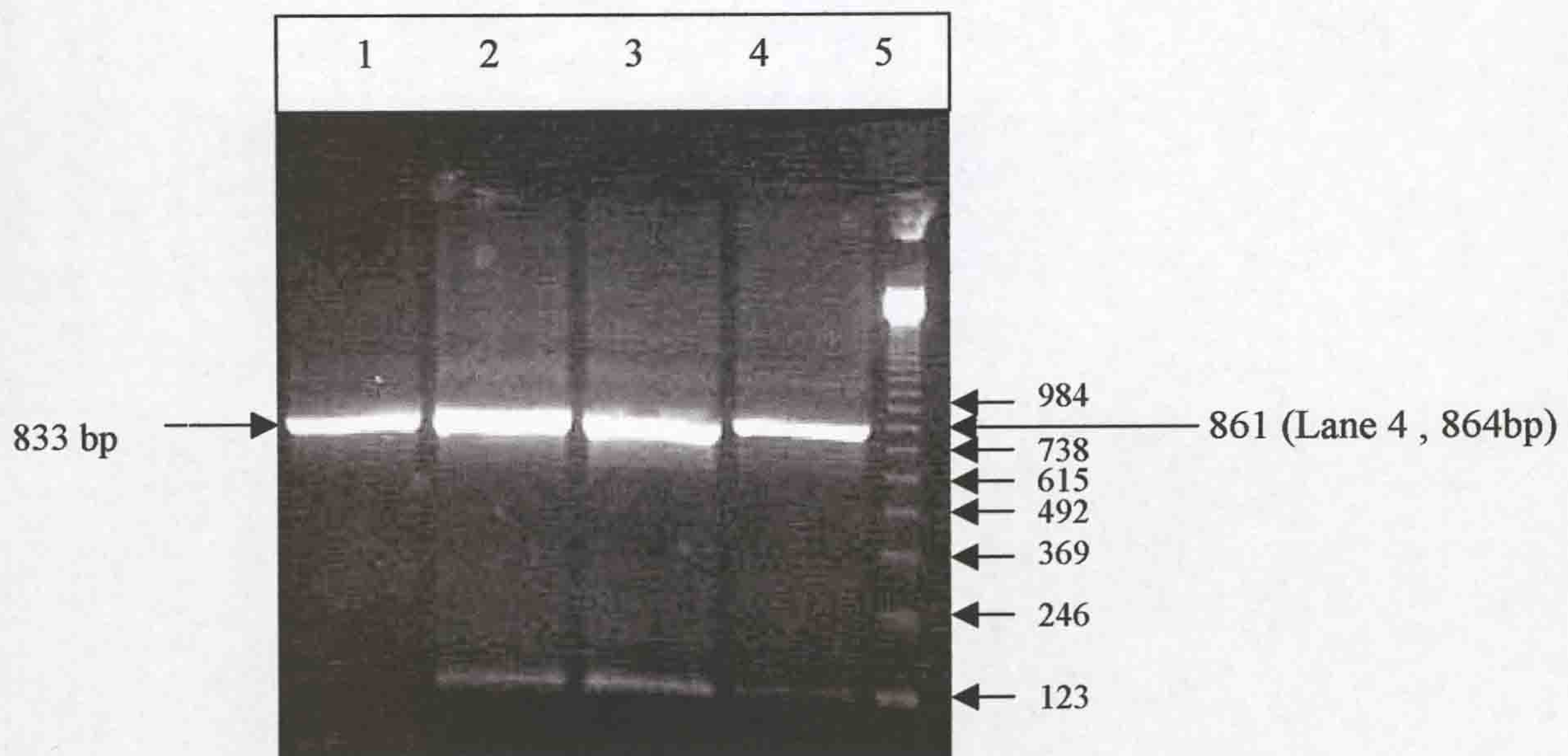


Fig 6.2b Visualisation of PCR product illustrating the different sizes generated after secondary PCR of selected samples from Thailand. Lanes 1-3 show samples of approximately 830-840bp. Lane 4 have a sample from the study of approximately 864bp.

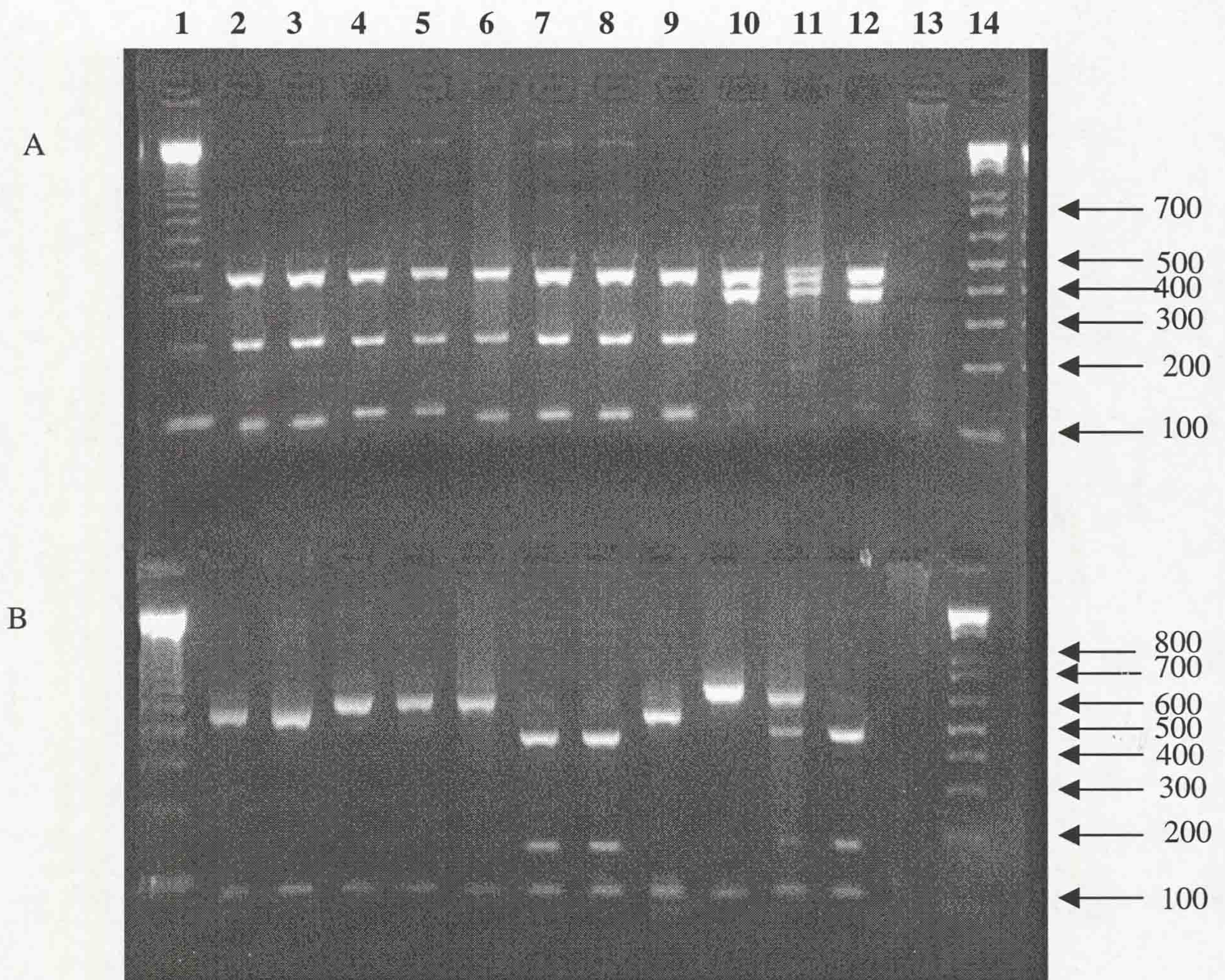
6.6.4 Restriction fragment length polymorphism (RFLP)

Amplification of the secondary fragment resulted in PCR products between 738 and 861bp on the 123bp molecular weight ladder. This corresponded to the expected 840-860 bp product of the target internal sequence of the 18S rRNA gene for *Cryptosporidium* species (Fig 6.2 a). Selected products were then visualised using larger volumes as shown in Fig 6.2b. Lane 1,2, and 3 had products approximately 830-840bp while lane 4 had a bigger product that was closest to the 861bp marker of the 123bp ladder. This showed the sample was non-parvum *Cryptosporidium*, possibly *C. felis.* To identify the genotypes the secondary products were digested with the *Ssp*I and *Vsp*I and visualised as shown in Fig 6.3 below.

Digestion with *Ssp*I shows a characteristic pattern of three major visible bands in both *C. parvum* and *C. meleagridis* and related species. These include a 449bp fragment, a 254 and 111-119bp bands (Lanes 2-9). Lanes 10-12 show non-parvum *Cryptosporidium* species with only two prominent fragments. The different genotypes were identified through further digestion with *Vsp*I. Lanes 2 and 3 showed fragments corresponding to *C. parvum* 'human' genotype of approximately 560bp, 104bp and a small one of approximately 70bp. Lanes 4-6 showed fragments identified as *C. parvum* 'bovine' genotype with fragments of approximately 625bp, and 104bp. Lane 7-8 showed fragments of approximately 450bp, 170bp and 104bp that corresponded to the pattern expected for *C. meleagridis*. Lane 9- had similar pattern to lane 2-3. Lane 10 showed the largest fragment approximately 730bp and a 100bp similar to the pattern seen in *C. muris* while lane 11 corresponded to *C. felis* with fragments of 658 and 104bp. Lane 12 showed a sample with fragments of approximately 470bp, 180bp

and 104bp. The fragment sizes of the isolate in lane 9 from the *Ssp*I digestion did not present a typical RFLP pattern while the *Vsp*I digestion pattern was closest to that of samples on lane 7 and 8, identified as *C. meleagridis*. The combination of the two patterns from both enzymes did not match any of the typical RFLP and no conclusive identification was made for this sample.

Fig 6.3 Restriction fragment length polymorphism (RFLP)



A= *Ssp*1 digestion; B = *Vsp*1 digestion

Fig 6.3 Visualisation of the restriction endonuclease digestion of the 18S RNA gene of *Cryptosporidium* species identified in the study. The upper gel show the results of restriction digestion with *Ssp*1 and the lower gel shows digestion with *Vsp*1. Lanes: 1 123bp marker; Lanes 2 shows the positive control; Lanes 3-12 show the different test samples of *Cryptosporidium* from the study; Lane 13 negative control; Lane 14 is the 1Kb marker.

6.6.5 Results of RFLP identification of the *Cryptosporidium* isolates in relation to source and HIV status

Results from the RFLP identified six different types of *Cryptosporidium* species and genotypes from all the five geographical regions in both HIV-infected and uninfected people. The summary of the different species and genotypes identified are shown in Fig. 6.4 and Fig 6.5. The highest numbers of isolates were identified as *C. parvum* 'human' genotype with a total of 62 of the 98 samples identified by RFLP from all regions. Others tentatively identified included 21 samples identified as *C. parvum* 'bovine' genotype, 8 *C. meleagridis*, 1 *C. muris,* 2 *C. canis* and 4 *C. felis* isolates. Occurrence of these species and genotypes by geographical source is shown in Fig 6.4 below. The highest diversity of the genotypes was in isolates from Thailand (Fig 6.4). These included 14/30 (samples that were identified by RFLP) *C. parvum* 'human' genotype, 4/30 *C. parvum* 'bovine' genotype, 7/30 *C. meleagridis*, 3/30, *C. felis*, and 2 *C. canis*. Extensive heterogeneity was observed in most *C. parvum* genotypes. All potentially zoonotic species and genotypes (except *C. parvum* 'human' genotype) were identified in HIV-infected patients (Fig 6.5).

Fig 6.4 Species and genotypes by RFLP in relation to sample source

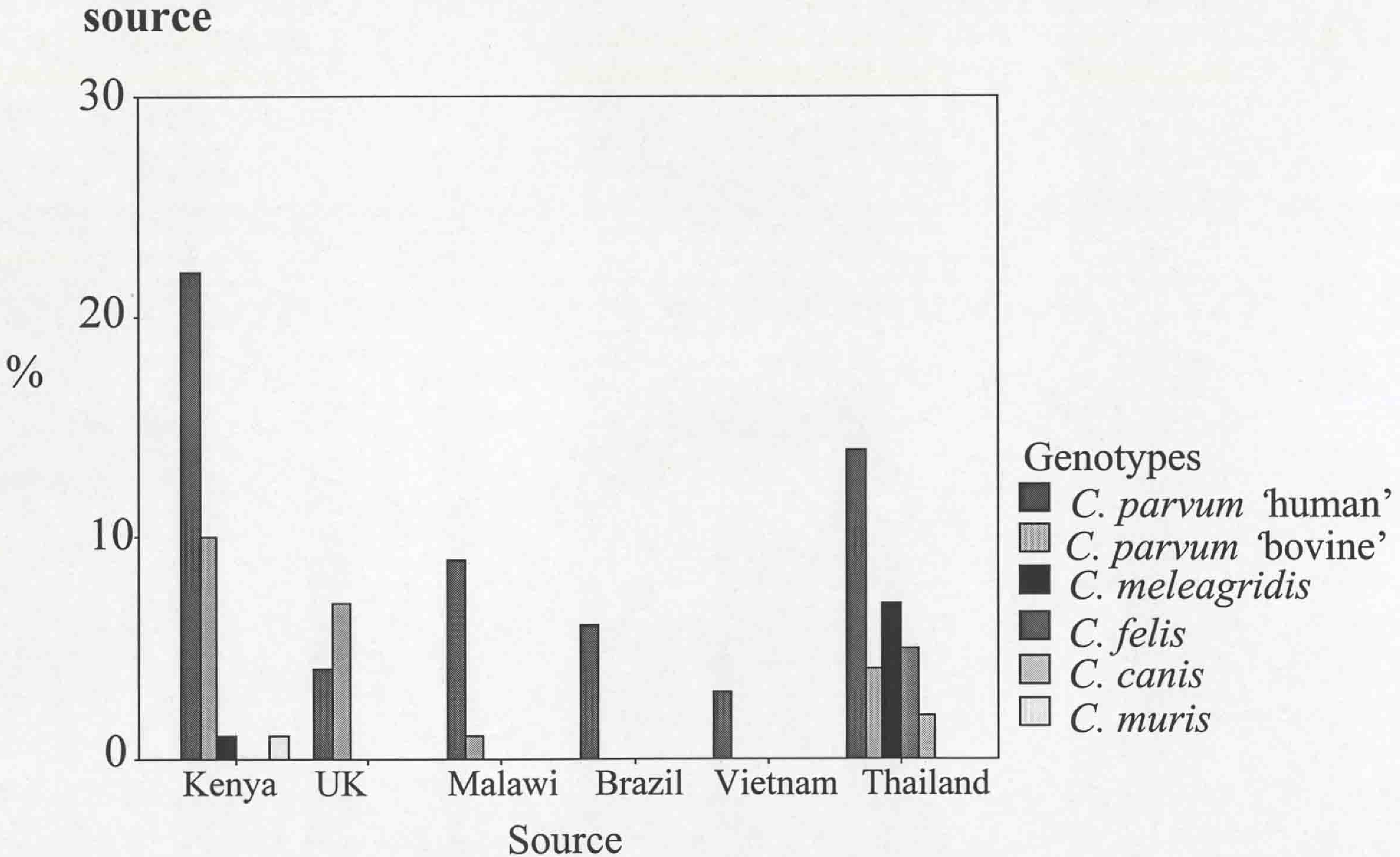
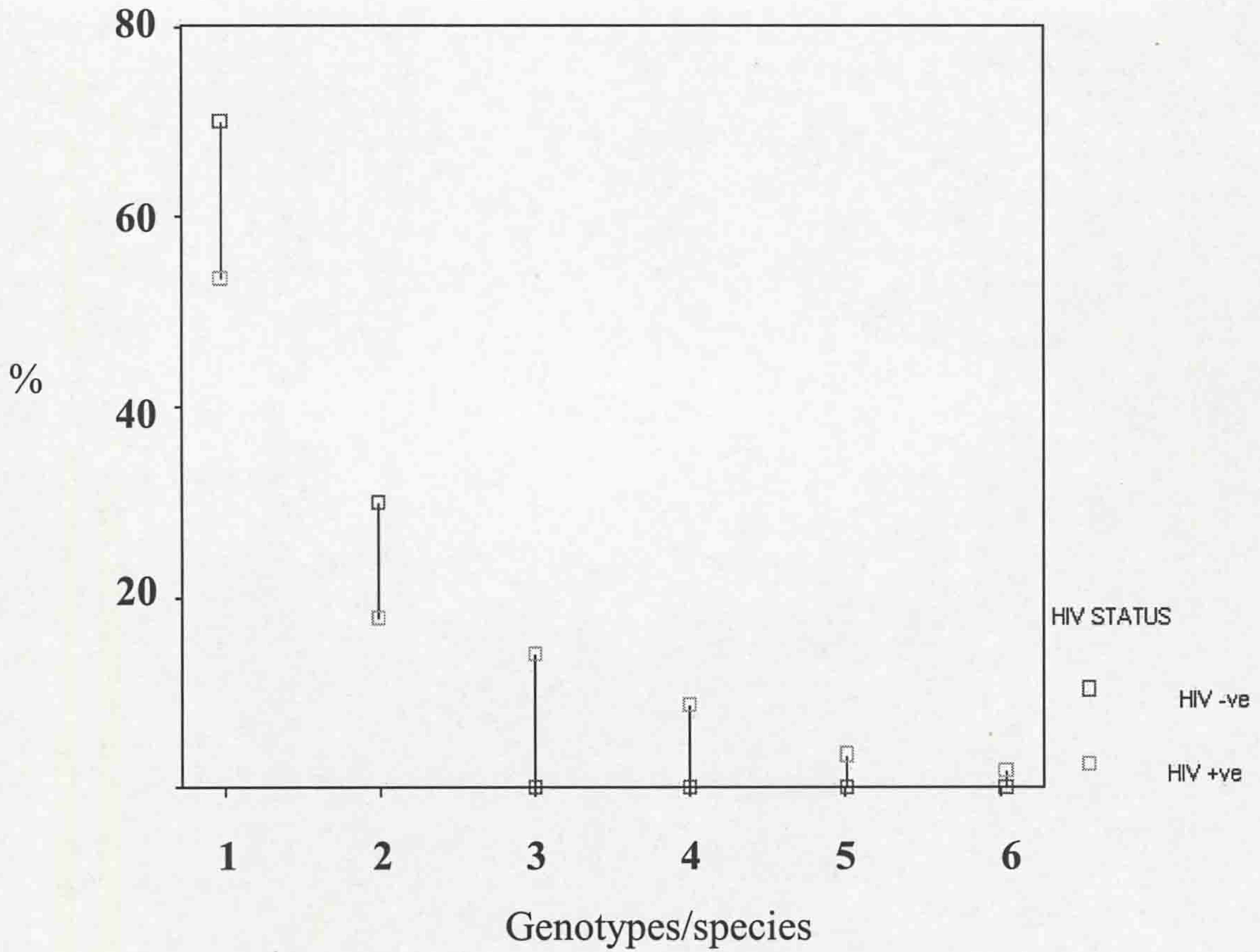


Fig 6.4 shows the proportion of isolates identified by RFLP from the different geographical regions.

Fig 6.5 Species and genotypes in relation to HIV status of patients



Genotypes/Species: 1=*C. parvum* 'human', 2=*C. parvum* 'bovine', 3= *C. meleagridis*, 4=*C. felis*, 5= *C. canis*, 6=*C. muris*

Fig 6.5 shows the occurrence of the different species and genotypes by HIV status. *C. parvum* 'human' and 'bovine' genotypes were most prevalent in HIV- uninfected people while the other potentially zoonotic species were all in HIV infected persons.

6.7 Summary of the genotypes identified by RFLP

Results from RFLP confirmed over 80% (83/98) of all samples were *C. parvum* 'human' and 'bovine' genotypes (Fig 6.4). The two genotypes were identified in samples from most regions. *C. parvum* 'human' genotype was the only one identified in available samples from Brazil and Vietnam The proportion of samples genotyped from HIV infected children was too small to make meaningful conclusions. 10 isolates from Kenya, and 2 from Thailand did not yield any PCR product. The widest variation of zoonotic genotypes was in isolates from HIV-infected Thai-patients. These included *C. parvum* 'bovine' genotype, *C. meleagridis* and *C. felis* while a small proportion (6 isolates from Thailand) could not be identified conclusively from their restriction patterns. The other zoonotic species isolated from Kenya was tentatively identified as *C. muris* since it has a similar restriction pattern to *C. andersoni*, a distinct species infecting cattle originally reported as *C. muris* (Xiao *et al.*, 1999b; Sreter *et al.*, 2000). Conclusive identification was therefore done using sequence analysis.

6.8 Discussion

Results from acid fast staining showed an overall 7% *Cryptosporidium* prevalence from the samples recovered in the MTCT cohort examined in the Chapter 2. HIV-infected children had the highest prevalence of over 15%, but their number was small (n=3). While acid fast staining was specific for *Cryptosporidium* as confirmed by PCR, it can be assumed that the prevalence is an underestimation since the stains were direct faecal smears that are likely to miss light infections or asymptomatic carriers (Ma and Soave, 1984). Earlier surveys from Kenya have reported less than

4% cryptosporidiosis in communities and in hospital patients (Simwa *et al.,* 1989; Estambale *et al.,* 1989). Prevalence among the HIV-infected people in the communities in the Kisumu/Busia cohort was lower (7%) than those found among HIV/AIDS patient in a previous study in Kenya. In one study in Kenya, a prevalence of 17% was recorded among patients with clinical AIDS (Mwachari *et al.,* 1998). Considering most of our participants did not have any apparent enteric disease, the prevalence may be representative of the occurrence of the organism in a rural community based on the sensitivity of the acid fast staining method used.

Acid fast staining has limitations in species differentiation of *Cryptosporidium* since most of the species and genotypes of the parasite are morphologically indistinguishable. This is the case for most *C. parvum* and related species (*C. meleagridis, C. felis, C. canis*) that infect the small intestines while *C. muris* that is distinctly larger in size and more ovoid than spherical is indistinguishable from *C. andersoni* (Fayer *et al*., 2000). The method is however, useful as a baseline screening method where *Cryptosporidium* organism is suspected. It is extensively used since clinical diagnosis is usually limited to genus rather than species of *Cryptosporidium*. Where specific identification is required, this method is unreliable and PCR and genotyping are necessary.

There were isolates positive with acid fast staining that had no PCR product. This problem was attributed to the method of faecal preservation and DNA extraction. Faecal samples preserved in potassium dichromate coupled with phenol/chloroform DNA extraction, yielded DNA that was a poor substrate for PCR in spite of the

presence of crude genomic DNA. Removal of potassium dichromate prior to PCR is essential (Johnson *et al.,* 1995) as traces of the preservatives could inhibit PCR. Our impression was that freezing of freshly collected faecal samples with no preservatives at -80^0C and consequent DNA extraction using the commercial kit gives the highest amplification rate for *Cryptosporidium.*

In at least one sample from Thailand, there was weak amplification and RFLP did not yield any observable bands for conclusive genotyping. In two other isolates from Thailand, there was no amplification despite repeated DNA extraction followed by PCR. Sequence polymorphism at this gene locus could cause amplification failure (Widner *et al.,* 1998; Widner, 1998). The presence of long T-repeat nucleotide segments in some *C. parvum* 'human' genotypes (up to 11T repeats) in the target fragment greatly reduces the sequence signal and could lead to amplification failure in regions beyond this segment (Xiao *et al.,* 1999c). Another factor could be due to the primers used. The primers in our study were adopted from those described by Xiao *et al.,* 1999b). These primers are based on conserved regions of the genus *Cryptosporidium* and while they are largely applicable, variation at the primer target region as may occur in novel species may result in lower specificity and amplification failure. PCR may also fail due to intrinsic shortfalls of the technique including excess inhibitors, minute differences in reagents or techniques that may require further standardisation or optimisation for specific species or genotypes.

Restriction fragment polymorphism was reliable in identification of the different species and differentiating *C. parvum* genotypes and *C. meleagridis.* The target gene

fragment used has advantages in that different species and genotypes are successfully amplified using two sets of primers and the resulting products are genus and species specific. This is unlike other gene targets such as the oocyst wall protein gene (COWP gene), TRAP-C1 gene, ITS gene and HSP70 gene. While these target genes are specific for each species, they require different primers for the various species and genotypes of C*ryptosporidium* (Xiao *et al.,* 1999b).

Restriction polymorphism demonstrated the multiplicity of the species and genotypes of *Cryptosporidium* infecting humans. The widest variety was from HIV infected patients from Thailand and Kenya. All samples from Thailand were from adult HIV infected patients. Our results showed almost 50% (14/31) of the isolates from Thailand were of zoonotic origin. The remaining six samples from the same area did not yield enough products for digestion. The resulting faint PCR products were then purified for cloning and sequenced to confirm the identity of the isolates. In one isolate from Thailand, there were clear visible bands but the isolate did not conform to any of the predicted fragment sizes. This too was cloned for subsequent sequencing.

It is not clear whether the extensive diversity in *Cryptosporidium* organisms infecting humans from Thailand is a typical occurrence, as we did not have non-HIV patients' isolates. Reports from Thailand from both HIV infected and un-infected persons have not previously differentiated specific species or genotypes isolated (Uga *et al.,* 1998; Punpoowong *et al.,* 1998; Manatsathit *et al.,* 1996; Wanke *et al*, 1999; Wiwanitkit, 2001). These reports identified only *C. parvum* or *Cryptosporidium* oocysts as the

pathogen isolated. The only other report of *Cryptosporidium* genotyping on isolates from Thailand identified 82.5% isolates as *C. parvum* 'human' genotype in the region (Tiangtip and Jongwutiwes, 2002). Others identified included *C. meleagridis*, *C. felis* and *C. muris*. This report and our data confirm a wide diversity of zoonotic species of *Cryptosporidium* parasites infect HIV-infected people in Thailand.

All our samples were of human origin. The number of potentially zoonotic isolates excluding *C. parvum* 'bovine' genotype recovered from humans worldwide is still few (Fayer *et al.*, 2000 summary of reports). Those from HIV infected persons in developing countries are fewer (Tiangtip and Jongwutiwes, 2002 summary of reports). In our study acid fast staining, PCR and RFLP showed the isolates were different species of *Cryptosporidium*. However, it was necessary to confirm their identities by sequencing since some of the predicted restriction patterns were inconclusive or distinctly different. The extensive intragentotypic heterogeneity at the 18S rRNA gene also means interpretation of restriction profiles may be inconclusive especially for new strains. Sequencing would therefore confirm the identity of the isolates and allow assessment of the isolates' genetic relatedness to published species and genotypes.

7.0 CHAPTER 7: SEQUENCING AND PHYLOGENY

7.1 Introduction

As stated earlier, sequencing was undertaken to confirm the identity of the isolates so far analysed. The method was also used to analyse the genetic diversity and phylogeny of *Cryptosporidium* species identified in the study based on the 18S rRNA gene fragment.

7.2 Materials and Methods

7.2.1 Sequencing

Samples with similar digestion patterns were grouped together. Representative samples were taken from each of these groups, from each geographical region, and from each category of either HIV infected or uninfected people. For efficient sequencing, samples were first purified by eluting the PCR product from 1.2% low melting agarose. DNA was purified from the low melting eluate using a commercial purification system (see below). The recovered DNA was then either sequenced directly or for samples yielding small amounts of DNA (less than 2ng), cloned into pGEMT-Easy plasmid vector (pGEMT-Easy Promega, Southampton, UK).

7.2.2 Purification of the secondary PCR product.

A 1.2% low melting agarose gel (Gibco BRL, UK) was prepared and 80μl of the secondary PCR product loaded into the wells. The product was run at constant 150V and visualised under UV with ethidium bromide. The product (approximately 840bp) was carefully sliced from the gel, put in a clean Eppendorf tube and weighed.

DNA was then eluted from the low melting agarose slice using a Sigma GenElute Mini-prep commercial kit (Sigma Chemicals, UK) according to the manufacturer's instructions. This method uses membrane columns that bind the DNA while allowing the dissolved agarose together with unincorporated primers and nucleotides to flow through. The membrane bound DNA was eluted with 50-70μl HPLC water.

7.2.3 Cloning

Principle

The procedure involves ligation of the DNA fragment into a plasmid vector containing markers (colour change, and antibiotic resistant β-lactamase gene). Transformed bacteria have antibiotic resistance and where the ligation is successful, bacteria colonies are white while the rest are blue. Transformed colonies are selected and grown in larger amounts of bacterial broth to yield enough copies of the test DNA. The DNA is then extracted from the transformed bacteria and purified for sequencing.

Procedure

All reagents were provided in the commercial pGEM-T Easy Cloning kit as were the instructions, extraction membranes and collection tubes. The eluted DNA was cloned into pGEM-T Easy Plasmid vector and transformed into comptenent *Escherichia coli* TG2 cells according to the manufacturers instructions using the following main steps.

7.2.3.1 Preparation of Competent TG2 cells

Escherichia coli TG2 were made competent by incubating with buffer RF1 on ice for 15mins, followed by centrifugation. The resultant pellet was incubated on ice with buffer RF2, for 30mins to 1 hour. RF1 and RF2 are as shown below.

RF1 Buffer

100 mM RbCl	(Sigma Adrich, UK)
30mM K acetate	(BDH)
10mM $CaCl_2$	(BDH)
50mM $MnCl_2$	(Sigma Adrich, UK)
15% Glycerol	(BDH)

RF2 Buffer

10mM MOPS	(Sigma M-1254)
75mM $CaCl_2$	
10mM RbCl	
15% glycerol	

The competent bacteria were aliquoted (200µl each) and stored at –20°C until use.

7.2.3.2 Cloning of PCR products into pGEM-T

PCR products were cloned into the pGEM-T vector by utilising the 3' A overhang introduced by Taq polymerase (Promega, pGEM-T vector system I). Methods were as described in the Promega manual. Briefly, approximately 100ng (3µl) of purified PCR product were incubated with 2X rapid ligation buffer, pGEM-T vector (50ng) and T4 DNA ligase (3 Weiss units; all provided), in a total volume of 10µl, for 1 hour on the bench.

7.2.3.3 Tranformation

After ligation, pGEM-T vector with the insert was transformed into competent TG2 cells by incubation on ice (1 hour), heat shocking (42°C, 45 seconds), incubation on ice (5 minutes), and growth at 37°C in Luria broth (LB - 10g Bacto-tryptone, 5g Bacto-yeast extract, 5g NaCl (1litre)) for 45 mins. Resulting bacteria were plated into LB-agar plates supplemented with 100 μg/ml Ampicillin (Sigma Adrich, UK), 80 μg/ml X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, (Calbiochem) and 200 μg/ml IPTG (isopropyl-β-D-thiogalactopyranoside, Sigma Adrich, UK), and grown at 37°C overnight.

pGEM-T carries an ampicillin resistance marker, and the PCR product is inserted into the galactosidase gene, disrupting its function. Hence, only bacteria containing vector with PCR product will grow as white colonies. Transformed colonies were screened by PCR with internal primers AL1598 and AL3032 described in section 6.4.1 above. A marked white colony was carefully picked with a pipette tip and used as the test DNA substrate in the secondary PCR. Colonies containing the vector with the target cloned PCR product were grown in 500mls of Luria broth, overnight in at 37^0C in a 200rpm shaker.

7.2.3.4 Recovery of the DNA from transformed bacteria

DNA was recovered from the transformed bacteria using the QIAprep spin miniprep kit, (QIAGEN, Crawsley, UK) according to the manufacturers instructions. The method involves pelleting the bacterial broth to recover the cells, which are then lysed

to release DNA. The mixture is passed through a prepared membrane filter, and washed several times with high salt buffers (all provided). DNA is then recovered using a spin column membrane that binds the DNA. This is then eluted from the membrane using 50-70μl HPLC water.

DNA concentration was then measured using Optic Density (260:280) in a Spectrophotometer (Biomate 3 Thermo Spectronic, Rochester NY USA). Approximately 1μg of test DNA was aliquoted into an Eppendorf, labelled, and sequenced with vector specific primers (M13-20, GTAAAACGACGGCCAGTGAG, M13-rev, GGAAACAGCTATGACCATG) using an ABI Automatic Sequencer (Lark Technologies, UK).

7.2.4 Sequence analysis

Sequences were read on Chromas version 1.45 (1996; C. McCarthy, Griffith University, Southport, Queensland, Australia). Manual editing and construction of consensus sequences was done using DNASTAR version 4.05 (1993-2000). Blast searches (WU-2 EMBL) were carried out to assess homology and sequence identity from the GenBank. Multiple alignments of the DNA sequences were by CLUSTALX (EMBL, Heidelberg, Germany) program and manual adjustments. Phylogenetic analyses were conducted using Phylogeny Inference Package (PHYLIP version 3.5c), (© J. Felsenstein and the University of Washington). Sequences were analysed using DNADIST followed by neighbor joining (NEIGHBOR, PHYLIP package). Further molecular evolutionary analyses were conducted using MEGA version 2.1 (Kumar *et al.*, 1993). Two thousands (2000) replica samplings were analysed for percentage

bootstrap values in a neighbour joining tree. Nucleotide diversity and divergence between the different species was assessed using DnaSP version3 (Rozas and Rozas, 1999) as described below (Section 7.2.4.1).

Sequences retrieved from the GenBank were AF093489 strain HCNV4, and AF159111 strain HCTX8 for the *C. parvum* 'human' genotype; AF093490 strain CpBOH, for *C. parvum* 'bovine' genotype; AF112574 strain CMEL for *C. meleagridis*; AF108862 strain Cat1 for *C. felis*; AF112576 strain CPD1 for the *C. parvum* 'dog' type also called *C. canis.* AF093498 strain, CMRH from rock hyrax for *C. muris,* and AF093496 for *C. andersoni* from cattle.

7.2.4.1 Nucleotide Diversity

The evolutionary relatedness among the different *Cryptosporidium* isolates was calculated using the Kimura –2 distances since the organism genome is AT rich (Kimura, 1980). The method is accurate in measuring the number of transitional and transversional substitutions per site for non-coding sequences such as the ribosomal RNA genes. Differences between any two sequences are measured in terms of number of nucleotide substitutions per site and deletions or insertions are excluded from the comparison. Genetic diversity and polymorphism was analysed according to the geographical source of the isolate and the HIV status of the patients.

Gene diversity is the measure of the probability that two alleles chosen at random from the population will be different from each other, thus is an estimate for genetic polymorphism. In long sequences, the likelihood that each has at least one or more

different nucleotide is high hence probability of gene diversity is close to 1. In such circumstances nucleotide diversity using Tajima's test (Tajima, 1989) is more appropriate. This is a statistical measure of total number of mutations assuming a 'neutral model prediction' (i.e. testing the hypothesis that all mutations are selectively neutral). It was appropriate for the fragments studied since the target sequence was of relatively long length (approximately 840bp) and being ribosomal RNA, it is a non-coding region.

7.2.4.2 Phylogenetic tree construction

Evolutionary relationships are illustrated with phylogenetic trees with nodes representing the taxonomic unit while the branches define the relationships among the units in terms of descent and ancestry. There are numerous methods of drawing phylogenetic trees. For nucleotide sequences data, the most common methods are the distance matrix method, maximum parsimony (MP), neighbour joining (NJ) and maximum likelihood (ML). In the distance matrix method the number of nucleotide substitutions between sequences are computed for all pairs of taxa and a phylogenetic tree is constructed by using an algorithm based on the functional relationships among distance values.

In maximum parsimony methods, the character states (nucleotide at a site) are used and the shortest pathway leading to these character states is chosen as the best tree. The NJ method finds the nearest 'neighbour' sequentially (through nucleotide homology) that when joined, minimizes the total length of the tree. In ML, the pattern of nucleotide differences at a site among all sequences involved is assessed and the

one with the 'largest maximum likelihood' preferred (Wen-Hsiung Li, 1997). The statistical validity of the tree is assessed by use of bootstrap, a computational technique that estimates the confidence level of the phylogenetic hypothesis (Felsenstein, 1985).

In this study the NJ tree was preferred in order to illustrate the order of isolates based on the nucleotide homology of the sequences. Both rooted and unrooted NJ trees were used. In the rooted tree, the most distant isolate was used as the out-group and distance was based from the selected isolate. In the unrooted, no out-group was defined and isolates clustered according to their relative genetic distances from each other.

7.3 Results sequence diversity and phylogeny

7.3.1 Cloning

Samples were divided into similar groups depending on the patterns of the restriction digestions. Representative samples were cloned as described above. Samples identified as non-parvum tentatively as *C. meleagridis* (7), *C. muris* (1), *C. felis* (3) and *C. canis* (2) and 10 -representative samples each from Kenya, Malawi, and Thailand, 5 from Brazil, 3 from Vietnam and 5 from UK were also cloned.

Fig 7.1 Transformed colonies

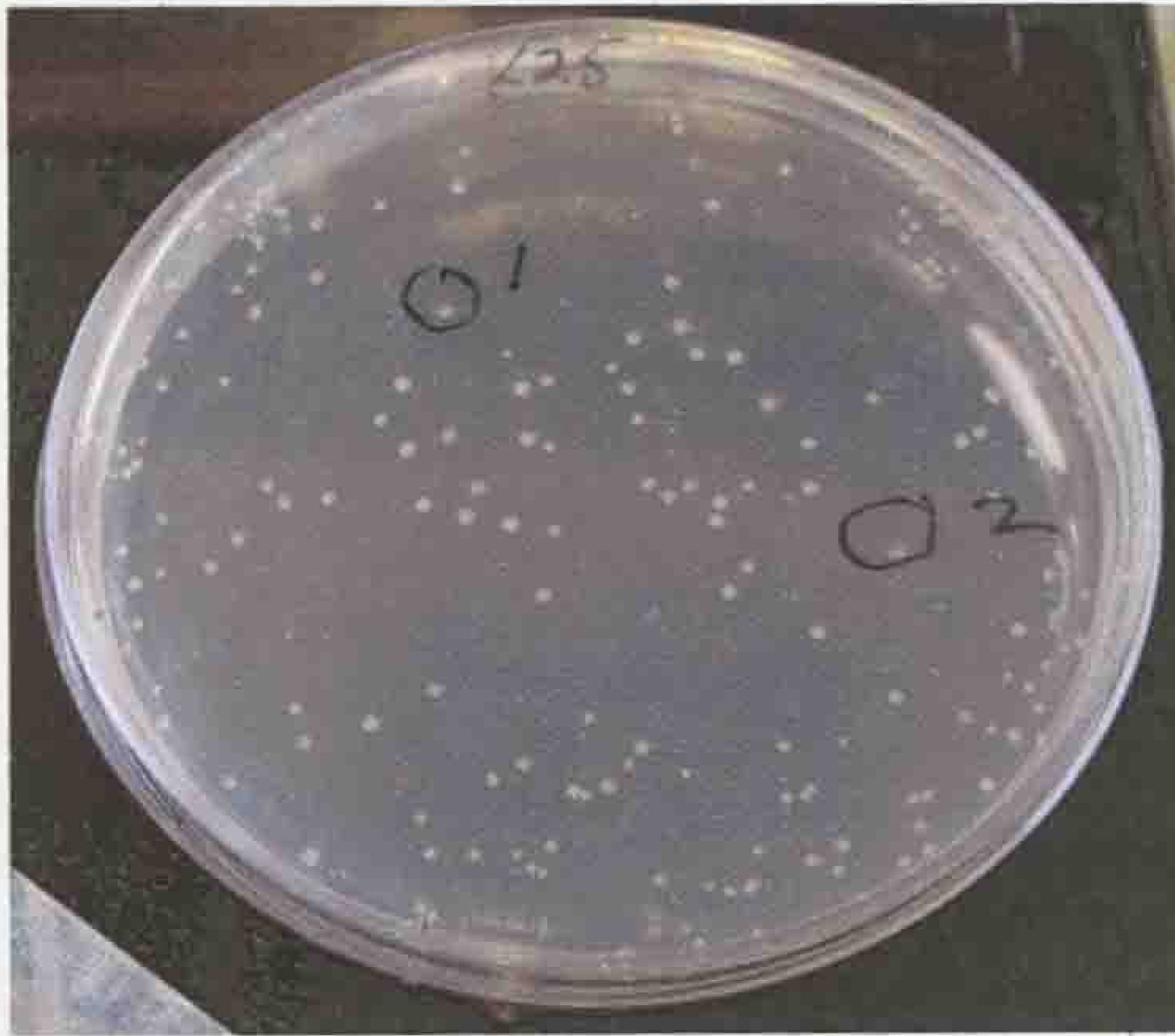


Fig 7.1 Example of an agar plate showing transformation of *E. coli* TG2 with pGEM-T Easy vector containing purified PCR products. Blue colonies contain a β-galactosidase gene. White colonies have a disrupted β-galactosidase gene. Plates contain ampicillin so only bacteria transformed by pGEMT Easy have ability to grow.

Fig 7.2 PCR products of screened white colonies of transformed bacteria

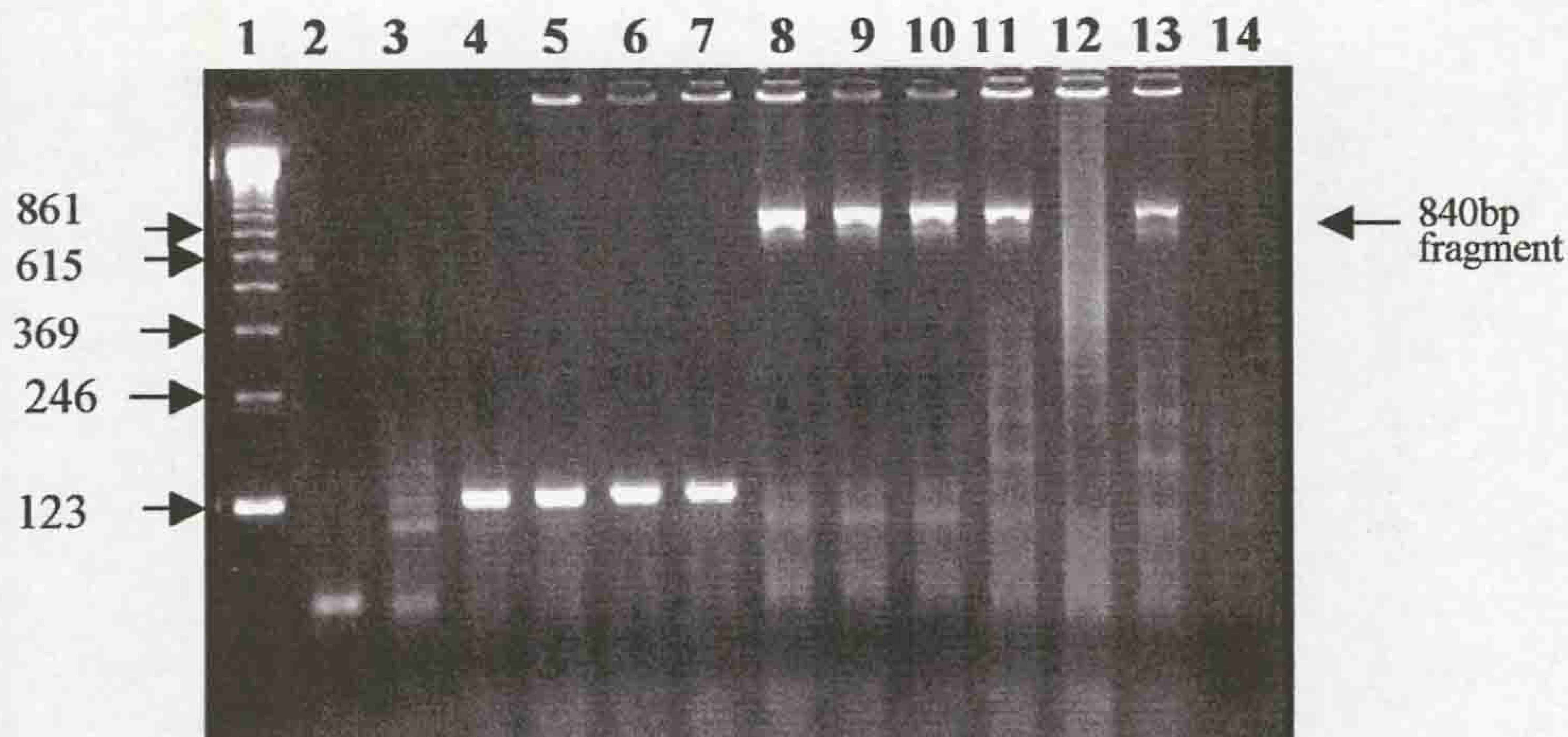


Fig 7.2 Visualisation of PCR product amplified using selected transformed colonies. Lane 1 molecular weight maker (123 bp) Lanes 2-7 no band at the expected PCR product size Lane 8-11. Lane 13 was a positive control while Lane 14 is the negative control.

Running secondary PCR reactions identified transformed colonies. A minute amount of the selected colony was used as the source of the test DNA in the PCR reaction. White colonies may occasionally have no insert as shown in Fig 7.2 (Lane 2-7). Those with the desired PCR product of approximately 840 bp (Lanes 8-11) were selected for purification and sequencing.

7.3.2 Sequencing Identity

All isolates were run on Blast search (WUBLAST-EMBL) to determine their identity and sequence homology to those in the GenBank. The Blast searches confirmed all sequences to be of *Cryptosporidium* genus with varying degrees of sequence homology to different species and genotypes in the GenBank. This corresponded to what was observed in acid-fast staining, PCR and restriction polymorphism that isolates were indeed *Cryptosporidium* organisms. As observed earlier, the highest diversity was in isolates recovered from HIV infected adult patients and specifically from Thailand. Restriction sites from the sequences were analysed in order to correlate with the observed RFLP fragments for specific species and genotypes, as shown below (Table 7.1).

Sequences used were deposited in the EMBL Nucleotide Sequence Database under the accession numbers AJ493191 to AJ493211 for isolates from Thailand. Sequences from the other Kenya, Malawi, Brazil, Vietnam and UK identified in the study were deposited under the accession numbers AJ493526 to AJ493528; AJ493030-AJ493542; AJ493544-AJ493549.

Table 7.1 Restriction sites and size of product from selected analysed sequences

Isolate	Species/ Genotype	PCR product	Fragments (bp)	
			*Ssp*I	*Vsp*I
Bz16/ K52	**C. parvum *'human'***	837	449, 254,111, 12,11	561,104,102,70
Vt13	*C. parvum* 'human'	834	449, 254, 108, 12,11	558,104,102, 70
K39	*C. parvum* 'bovine'	**831**	449,254,119,9	625,104,102
Th13	*C. parvum* 'bovine'	834	449,254,117,12	628,103,102
Th22	*C. meleagridis*	**835**	**462**, 254, 108, 11	**458**, 171, 104,102
K53	*C. meleagridis*	833	449,254,108,11,11	456,171,104,102
Th3	*C. meleagridis*	833	449, 254,108,11,11	456,171, 104, 102
K33	*C. muris*	833	448, 385	731, 102
Th7	*C. felis*	864	459, 391, 14	**658,** 104, 102
Th26	*C. felis*	864	460, 389, 15	**477**,181,104,102

Numbers in bold show sequences with unique fragment sizes compared to published strains.

Samples are coded according to the country of origin of the sample with Bz=Brazil,

K=Kenya, M=Malawi, Th=Thailand, Vt=Vietnam, UK=United Kingdom.

Sequences were identified depending on their degree of homology with published strains. Isolates were considered 'different' if they had at least one nucleotide difference from the closest sequence from the GenBank. Results are presented as a summary of the percentage nucleotides per sequence fragment (Appendix 5). None of the *C. meleagridis* and *C. felis* showed 100% identity to any of the published sequences. Isolate K33 was confirmed to be *C. muris* showing 100% sequence identity with *C. muris* isolate from rock hyrax strain CMRH (Acc No AF093498). Another isolate that failed to produce discernable PCR product was identified as C. *canis* with 100% sequence identity with published strain CPD1 (*C. parvum* 'dog' genotype; Acc No. AF112576). Three isolates from Thailand were identified as *C. parvum* 'human' genotype while no identification was made on two isolates as both the PCR and cloning failed to yield any discernable bands.

A significant finding was observed in the variation within the *C. meleagridis* and *C. felis*. One *C. meleagridis* isolate (Th22) had two base insertions at 553 and 577 nucleotide positions (based on the positions in the complete 18S rRNA gene) resulting in an 835bp fragment. Comparison of the *C. felis* sequences also revealed significant differences as reflected in the restriction endonuclease digest. One isolate (Th26) had an extra base A at position 671 that resulted in the creation of an extra *Vsp*I restriction site. On cleavage of the amplicon, a different restriction endonuclease pattern was found, with the largest amplicon significantly smaller (477bp) than the typical 658 bp fragment yielded by the other *C. felis* isolates.

7.3.2.1 Nucleotide Diversity

Restriction polymorphism and sequence homology identified five species of *Cryptosporidium* namely *C. parvum* 'human' and 'bovine' genotypes, *C. meleagridis, C. felis, C. canis* and *C. muris*. The 18S rRNA gene locus exhibits heterogeneity within organims but the extent of sequence and nucleotide diversity in the different species has not been fully analysed. An attempt was therefore made to analyse in detail the extent of nucleotide divergence between the sequences within and between the species identified.

Nucleotide diversity and mutation rates were assessed using DnaSP (version 3) as indicated earlier. Nucleotide diversity is the measure of the average number of nucleotide substitutions per site between sequences. Each pairwise comparison of all sites in the test sequences was obtained and a final average for all the values gives the nucleotide diversity within each species. The first analysis was on diversity within each species. Results revealed variation was highest in the *C. parvum* 'human' genotype group (Table 7.2 below). There were 19 distinct sequences out of 31 identified as *C. parvum* 'human' genotype. Within this group, 31 segregating sites (S) with a total of 32 mutations (Eta) were detected. Nucleotide diversity (Pi) for the 31 sequences was 0.00398. The genotype showed the highest mutation rate per nucleotide site (Theta) of 0.01036. The results of nucleotide diversity for the other species and genotypes are as shown in Table 7.2 below. At least four different sequences are required for the estimation of Tajimas D-value. As such no values were obtained for *C. felis* while extra sequences identified as *C. muris* used were obtained from the GenBank.

Table 7.2 Nucleotide diversity and mutation rates of *Cryptosporidium* genotypes at the 18S rRNA gene fragment

Species	No of Seq	Total sites	S	Eta	Eta (s)	Theta /Eta (s)	Pi	Theta /site	Theta /seq	Tajimas D-value	*p*< 0.05
C.parvum 1	31	773	31	32	27	26.13	0.00398	0.01004	7.76	-2.174	0.01
C.parvum 2	10	771	11	11	8	6.67	0.00562	0.00625	4.82	-0.608	0.10
C. meleagridis	8	776	10	10	8	6.86	0.00417	0.00526	4.65	-1.109	0.10
C.felis	2	814	4	4	8	6.67	0.00498	0.00498	4	-	-
C.muris	4	773	8	8	7	5.25	0.00539	0.00565	4.36	-0.446	0.10

S = Number of polymorphic sites
Pi = Nucleotide diversity
Eta = Total number of mutations
Eta (s) = Total number of singleton segregating sites
Theta/Eta (s) = mutation rate per singleton site
Theta/site = mutation rate per nucleotide site
Theta/seq = mutation rate per sequence
Tajima's D value = neutrality tests (all mutations are neutral)
p value = level of significance in differences between sequences (within species)

The results demonstrate the extensive diversity within *C. parvum* 'human' genotype where sequences were significantly different from each other (Tajima's D value – 2.174, *p*=0.01). This was also demonstrated in the high mutation rate per sequence of 7.76 compared to the other species with values of around 4. The extensive nucleotide diversity in this genotype was mainly due to the high number of singleton mutations (Eta(s) =27) with a consequent mutation rate at singleton sites (Theta per Eta (s)) of 26.13.

The mutation rate per singleton site was much lower in other species including the *C. parvum* 'bovine' genotype at approximately 6 (Table 7.2). In all the other genotypes, there was no significant difference between sequences (p=0.10).

Nucleotide diversity between species was also analysed using the same parameters. The results are presented in Table 7.3 below.

Table 7.3 Divergence between species based on nucleotide diversity

Comparison of species		PS	Eta	K	Pi	FD	NDP	NSP
C.p. human	C.p bovine	42	43	3.940	0.00514	2	6.231	0.00812
	C. meleagridis	44	46	4.787	000621	4	8.604	0.01116
	C. felis	51	53	5.451	0.00706	22	23.823	0.03086
	C.muris	78	80	12.440	0.01670	42	49.677	0.06668
C. p bovine	*C. meleagridis*	25	26	7.026	0.00914	5	9.881	0.0128
	C. felis	30	33	12.5	0.01623	21	23.667	0.03074
	C. muris	58	60	27.422	0.03681	43	48	0.06443
C.meleagridis	*C.felis*	33	34	11.778	0.01520	22	25.286	0.03263
	C. muris	60	60	2.255	0.03505	44	48.571	0.06485
C. muris	*C. felis*	71	71	36.467	0.04748	59	64.75	0.08431

PS= polymorphic sites
k= number of nucleotide differences between sequences
Eta=Total number of mutations
Pi = Nucleotide diversity
k = average number of nucleotide differences between sequences
FD=Fixed differences between populations
NDP= average number of nucleotide differences between genotypes
NSP=Average number of nucleotide substitutions per site between genotypes

Comparison of sequences from the different species at this locus suggested close relationship between *C. parvum* 'human' and 'bovine' genotypes with *C. meleagridis*. In spite of the high number of mutation sites between *C. parvum* 'human' and 'bovine' genotypes (Eta = 42) and between the former and *C. meleagridis*, there were only 2 and 4 fixed differences respectively. Fixed differences refer to the number of mutations occuring between any two sequences from the different groups that are maintained in all the pairwise comparisons. The total number of mutation sites (Eta) was less between *C. parvum* 'bovine' genotype and *C. meleagridis* (Eta = 26) than between *C. parvum* 'human' genotype and *C. meleagridis* (Eta = 46) but the fixed differences in the two groups were close (5 and 4 respectively). Distances between

species appeared to be based more on the number of fixed differences than on any other factor. However, this appeared to vary when *C. felis* was compared to *C. muris*. While the highest number of mutation sites were observed between *C. parvum* 'human' geotype and *C. muris* (Eta = 80), the two had less fixed differences (FD= 42) compared to those between *C. muris* and *C. felis* where Eta = 71 and FD=59 as shown in Table 7.3 above.

Sequence diversity was further assessed using the evolutionary distances between the sequences as shown in Table 7.4. The results showed *C. muris* was the most genetically distant isolate with a divergence of at least 6% to all the other species. The results also suggested *C. canis* was closer to *C. meleagridis* (about 1.9%) than to *C. felis* (2.9%). The analysis showed the widest distance was between *C. felis* and *C. muris* that were over 7%. This was in agreement with the earlier results from nucleotide diversity where the two had the largest number of fixed differences shown (Table 7.3 above).

Distances within the *C. meleagridis* species (that is between the different isolates identified as *C. meleagridis*) were comparable to those between the *C. parvum* 'human' and 'bovine' genotypes (0.4-0.5%). Inspite of the variation observed within *C. parvum* 'human' genotype group, samples within the group showed minimum genetic distances of 0.1-0.3% (Table 7.4). The variable sites of representative sequences used both in the nucleotide diversity assessments (Table 7.2 and Table 7.3) and evolutionary distance analyses (Table 7.4) are presented in Table 7.5 below. Parsimonic informative sites are presented in bold, while similar nucleotides are left

as dots. The high number of singleton mutations is apparent within the *C. parvum* human genotype group in samples numbered 1-19. Samples numbered 20-23 are the different *C. parvum* 'bovine'genotype while 24-30 was *C. meleagridis*. Samples number 31 was identified as *C. canis* while number 32 to 34 were *C. felis*. The last sample number 35 was identified as *C. muris*.

Sequences were aligned according to BZ16 isolate that had 100% identity with HCNV4 *C. parvum* 'human' genotype (Table 7.5). Twelve other samples from all geographical regions were similar to this sequence. Polymorphic sites demonstrated the diversity among the species from *C. muris* that is the most distant, to *C. felis, C. canis*, *C. meleagridis* and lastly, *C. parvum* group. A transitional switch of T to C on site 604, was conserved in *C. parvum* 'human' isolates from Thailand (Th18), Malawi (M15) and Vietnam (Vt13). The mutation was also conserved in the *C. parvum* bovine types, *C. meleagridis*, and *C. muris*.

An estimate based on the variance of the average number of nucleotide differences between pairs of sequences was done (DnaSP version3). This gives the minimum number of recombination events in the history of the samples. 36 sites had at least four gametic types of combination. Of these five recombinant events were detected at sites 45 / 119; 119 / 517; 517 / 553; and 680 / 780 (Table 7.5).

Table 7.4 Evolutionary genetic distances among selected *Cryptosporidium* species

Results: *C. parvum* 'human' genotypes (Bz16) used as consensus

Distance method : Nucleotide per 100 bases: Method, Kimura 2-parameter [Pairwise distances] Standard Error estimated by bootstrap method

(Replications = 500 and random number seed = 14811, Mega2.1)

Species	Type		Bz16	M8	UKm	K52	Vt13	Th2	K4	M14	K53	Th22	Th15	Th1	Th27	Th3	Th7	Th26	Th34
C. parvum	H	Bz16	-																
C. parvum	H	Mw8	0.1																
C. parvum	H	UKm	0.1	0.3															
C. parvum	H	K52	0.1	0.3	0.3														
C. parvum	H	Vt13	0.3	0.4	0.4	0.4													
C. parvum	H	Th2	0.1	0.3	0.3	0.3	0.4												
C. parvum	B	K4	0.4	0.5	0.5	0.5	0.7	0.5											
C. parvum	B	Mw14	0.3	0.4	0.4	0.4	0.3	0.4	0.4										
C. meleagridis		K53	0.8	0.9	0.9	0.9	0.8	0.9	1.2	0.8									
C. meleagridis		Th22	0.7	0.8	0.8	0.8	0.8	0.8	1.1	0.8	0.5								
C. meleagridis		Th15	0.8	0.9	0.9	0.9	0.8	0.9	1.2	0.8	0.5	0.5							
C. meleagridis		Th1	0.7	0.8	0.8	0.8	0.7	0.8	1.1	0.7	0.4	0.4	0.4						
C. meleagridis		Th27	0.7	0.8	0.8	0.8	0.7	0.8	1.1	0.7	0.4	0.4	0.4	0.3					
C. meleagridis		Th3	0.5	0.7	0.7	0.7	0.5	0.7	0.9	0.5	0.5	0.5	0.5	0.4	0.4				
C. felis		Th7	2.3	2.6	2.6	2.6	2.7	2.6	2.7	2.7	2.4	2.3	2.4	2.3	2.3	2.4			
C. felis		T26	2.3	2.4	2.4	2.4	2.6	2.4	2.6	2.6	2.3	2.2	2.3	2.2	2.2	2.3	0.1		
C. canis		Th34	2.3	2.4	2.4	2.4	2.3	2.4	2.7	2.3	2.0	2.0	2.0	1.9	1.9	2.0	3.0	2.9	
C. muris		K33	6.3	6.4	6.4	6.4	6.3	6.3	6.7	6.3	6.1	6.1	6.1	6.0	6.0	6.0	7.3	7.2	6.7

H= Human Genotype, B= Bovine genotype

Isolate identification Bz = Brazil, UKm = United Kingdom (monkey isolate) Mw = Malawi, K = Kenya, Vt = Vietnam, Th = Thailand

7.5 Summary of Variable sites for selected sequences. Number of Variable sites=97. Bold positions show Parsimonic sites

```
                     11 1111222222 2222222233 3333333444 4444444444 4444445555 5555555555 6666666666
              144444512 3379112234 5566668800 1125789112 2233333445 6677770011 5556788999 0122223577
             5903458695 0875161360 1805697867 0122408261 2301257018 2512687827 3459067038 4924573801
1.Bz16       TCAATTTTAG ATAAAAATTA AATTGTTAAA ATGTTTCTAA TTAAATTGAA TTAAAAGTTT ATACCTTATA TATAGAATTC
2.Th24       .......... .........G .......... .......... .......... .......... .......... ..........
3.M8         .......... .......... .......... ..A....... .......... .......... .......... ..........
4.K46        .......... .......... .......... ...C...... .......... .......... .......... ..........
5.Bz18       .......... .......... .......... ......A... .......... .......... .......... A.........
6.Th35       .......... .......... .......... .......... .......... .....G.... .......... ..........
7.M6         .......... .C........ .......... .......... .......... .......... .......... ..........
8.UKM        ......C... .C........ .......... .......... .......... .......... .......... ..........
9.K24        .......... .......... .......... .......... ....G..... .......... .......... ..........
10.M11       ......C... .......... .......... .......... .......... .......... .......... ..........
11.Th30      .......... .......... G......... .......... .......... .......... .......... ..........
12.M1        .......... .......... .......... G......... .......... .......... .......... ..........
13.M10       .......... .......... .......... .......... .......... .......... .......... ..........
14.K7        .......... ...G...... .......... .......... .......... .......... .......... ..........
15.K52       .......... G......... .......... .......... .......... .......... .......... ..........
16.Th18      .......... ....G..... .........G .......C.. .......... .......... .......... C.........
17.M15       .......... .......... .......... .C..C..... .......... .......... .......... C.........
18.Vt13      .......... .......... .......... .......... .......... .......... .......... C.........
19.Th2       .......... .......... .......... .......... .......... ........C. .......... ..........
20.M14       ...T.A.... .......... .......... .......... .......... .......... .......... C.........
21.Th13      ...T.A.... .......... .T........ .......... .......... .......... .......... C.........
22.K4.seq    ...T.A...A .......... .......... .......... .......... C......... .......... ..........
23.Cpbksu-1  ...T.A.... .......... .......... .......... .......... C......... .......... C.........
24.Cmel      .......... .......... .......... .......... ..T....... .......... .......... C...A.....
25.Th3       .......... ........C. .......... .......... ..T....... .......... .......... C...A.....
26.K53       .......... .....C.... .......... .......... ..T....... .......... .......... C...A.....
27.Th22      .......... .......... .......... .......... ..T....... .......... .......... G...A.....
28.Th15      .......... ..G....... .......... .......... ..T....... .......... .......... C...A.....
29.Th1       .......... .......... .......... .....C.... ..T....... .......... .......... C...A.....
30.Th27      .......... .......... .......... .......... ..T....... .......... .......... C...A.....
31.Th34      .T.T...... .......... ...CA..G.. .......... ..T....A.. .........C T.TAG..... C..GA....T
32.Th7       .....A..T. .......... ...C...... .........C C.T....TTG G.....TA.. T.T...A... ....A.....
33.Th26      .....A..T. .......... ...C...... .........C C.T....TTG G.....TA.. T.T...A... ....A.....
34.cat1      .....A..T. .......... ...C...... .........C C.T....TTG G.....TA.. T.T...A... ....A.....
35.K33       GTGTC.CCT. ......GC.. .GCC.CG.G. ........G. .CT.TCA.G. .CCCC.A.GC .AC..G.GCG CGC.AGGCC.
```

```
                6667777777 7777888
                8890000123 4789011
                0573459610 8603823
1.Bz16          ATAAAAAATA AATTTAA
2.Th24          ..C....... ..C....
3.M8            .......... .......
4.K46           .........G .......
5.Bz18          ....G..... C......
6.Th35          .......... .......
7.M6            .......... .......
8.UKM           .......... .......
9.K24           .......... .......
10.M11          .......... .......
11.Th30         .......... ....C..
12.M1           .......... .......
13.M10          ........C. .C.....
14.K27          .......... ......G
15.K52          .......... .......
16.Th18         .......... .......
17.M15          ....G..... .......
18.Vt13         ....G..... .......
19.Th2          .......... .......
20.M14          .......... .......
21.Th13         .......... .......
22.K4           .......... .......
23.Cpbksu       .......... .......
24.Cmel         .......... .......
25.Th3          .......... .......
26.K53          .......... ...C...
27.Th22         .....G.... .......
28.Th15         .A........ .......
29.Th1          .......... .......
30.Th27         .......G.. .......
31.Th34         .......... .......
32.Th7          T......... ..C....
33.Th26         T......... .......
34.cat1         T......... .......
35.K33          ...G..C... .....G.
```

B16 isolate (similar to published HCNV4 strain) was used as consensus sequence. 1-18 *C. parvum* 'human' type, 19-23 *C. parvum* 'bovine' type, 24-30 *C. meleagridis*, 31-*C. canis*, 32-34-*C. felis*, 35-*C. muris*.

7.5

7.4 Phylogeny of *Cryptosporidium* parasites

Figure 7.3 and 7.4 show the phylogenetic relationship between the *Cryptosporidium* samples based on the hypervariable region of the 18S rRNA gene. In the first analysis, *C. muris* was used as the out-group (Fig 7.3), as the species showed 86% or less sequence homology (>6.1% distance to all isolates). In the neighbor-joining phylogram samples were arranged in serial order through their relative sequence homology to each other while branch lengths represented the genetic distance between any two samples. Anchoring the tree on *C. muris*, *C. canis* emerged earliest followed by *C. felis* with high bootstrap values of over 71% (Fig 7.3). *Cryptosporidium canis* had a much shorter branch while *C. felis* had the longest branches among all the groups in this phylogram. The next clade was that of *C. meleagridis* and *C. parvum* emerging as sister groups with high bootstrap values of 87%. Heterogeneity with the *C. meleagridis* group was illustrated with intragenotypic variations with full statistical reliability (bootstrap values of 86% and 60%).

The two genotypes of *C. parvum* separated with full statistical reliability (bootstrap 91%). There were two clusters within *C. parvum* 'bovine' genotype, the first one included isolates from Kenya, Malawi, UK and Thailand while the other was mainly from isolates from Kenya (bootstrap value 82%). This second group clustered with the published KSU-1 strain 'gene B'. One sample (K39) appeared to take different positions during the tree construction with an unusually long branch. The result together with homology from the WUBlast confirmed the earlier observations that it was closest to *C. parvum* 'bovine' genotype CpbKSU identified as 'gene' B a variant of the SSU gene.

Cryptosporidium parvum 'human' group produced two clusters separating with high bootstrap value 86%. The diversity illustrated the extent of heterogeneity within the *C. parvum* 'human' genotype that was maintained in all regions and in both HIV infected and uninfected persons. The 'human' genotype group also included one isolate recovered from a monkey in a zoo in UK. This isolate was closest to one recovered from Malawi. Where human group clustered clearly into two groups variation within each group had low bootstrap support.

Fig 7.3 Phylogeny of *Cryptosporidium spp* by a rooted NJ tree

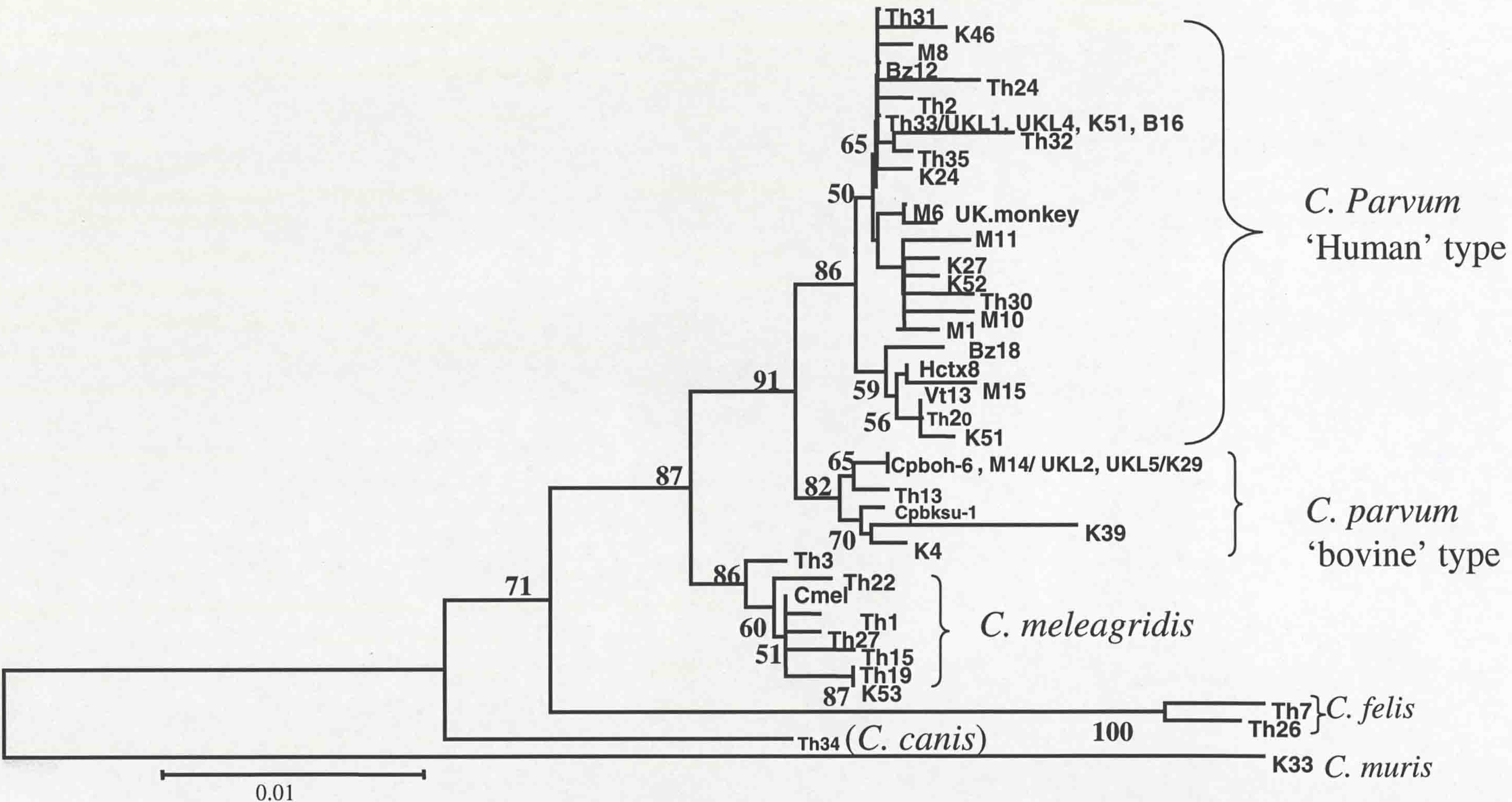
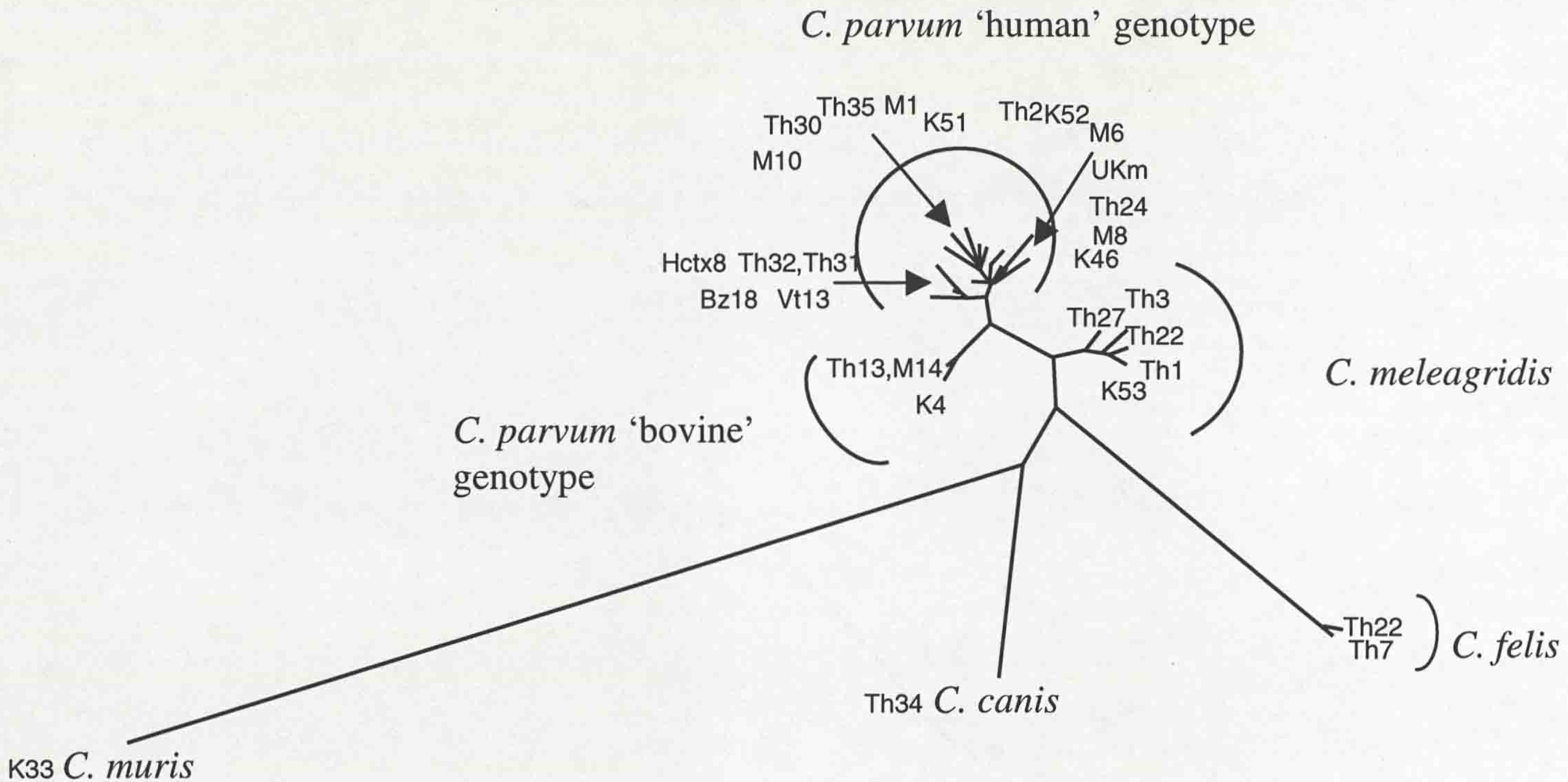


Fig 7.3 shows the phylogenetic relationships of the isolates from all regions in the study including published strains (Cpboh, Hctx8, Hcnv4, Cmel). Bootstrap values shown >50%. K33 (*C. muris*) was used as an out-group

An unrooted NJ phylogram illustrated further, the relationships of the isolates (Fig 7.4 below). The results were similar to earlier observations confirming the different genotypes identified in the study and the extensive intragenotypic variations within the *C. parvum* species. The phylogram showed the distribution of the genotypes from the five regions, with Thailand showing the largest diversity of isolates. The distance between *C. muris* and the other isolates with *C. canis* branching earliest followed by *C. felis* that again, was the furthest from *C. muris*. The close relationship between *C. meleagridis* and *C. parvum* group was demonstrated

Fig 7.4 Phylogenetic relationships of *Cryptosporidium* isolates using unrooted neighbour joining tree



7.5 Discussion

Sequences analysis and phylogeny confirmed the identities of the isolates recovered. Our study was the first report on human infection with *C. muris* identified using microscopy and molecular analysis (Gatei *et al*., 2002). The study is also the first, to the best of our knowledge, to report the occurrence of the *C. parvum* 'bovine' genotype and *C. canis* in HIV patients in Thailand. It is also in agreement with a recent report that demonstrated the presence of the other zoonotic species of *Cryptosporidium* including *C. felis* and *C. meleagridis* in HIV patients in Thailand (Tiangtip and Jongwutiwes, 2002). The occurrence of novel species in humans was highest among HIV infected patients. Still, *C. parvum* 'human' genotype as a single subtype was the most common in both HIV infected and un-infected patients.

While the largest intragenotype variation was within the *C. parvum* 'human' genotype, diversity did not appear to vary with geographical origin of the isolates nor with age of the patient or HIV status. Single nucleotide site changes occurred along the length of the gene fragment regardless of the geographical origin of the sample. There was less variation within the *C. parvum* 'bovine' genotype with most isolates from Kenya, Malawi, Thailand and UK showing 100% sequence identity. Heterogeneity appeared to be in the amplification of 'gene B' that occurs in *Cryptosporidium* organism. Isolates that amplified 'gene B' were from HIV infected adult patients from Kenya and showed a characteristic 3-base deletion (resulting to 831bp fragment compared to 834bp in the other isolates). Gene 'B' of the 18S rRNA shows considerable nucleotide differences with gene 'A'. The latter occurs in more copies at a ratio of 4:1 among the different alleles of the 18S rRNA positions in the

five chromosomes of the organism (Le Blancq *et al.*, 1997; Feng *et al.*, 1998; Spano *et al.*, 1999; Xiao *et al.*, 1999c).

There was less variation in the other species appearing to be single nucleotide changes among the *C. meleagridis* isolates. This species also exhibit heterogeneity at the 18S rRNA gene locus. The longer sequence fragment may therefore represent a variation within the organisms rather than a different strain. However, the heterogeneity observed in *C. felis* was significant. Insertion of the 'A' base resulted in the creation of a new cleavage site, and therefore a different restriction profile from the other *C. felis* isolates. This is significant as restriction digestion of the 18S rRNA gene is used in diagnosis and epidemiological mapping. Identification of 'novel' species in both humans and animals is on the increase (Fayer *et al.*, 2000). Our results indicate it is necessary to confirm the identity of isolates that may have atypical profiles by sequencing.

Previous phylogeny of *Cryptosporidium* has applied mainly isolates from natural hosts with the zoonotic strains derived from the respective animal sources. Isolates identified as zoonotic species in our study were all from human sources. Evolutionary distances were similar to those observed in other studies where animal isolates were used (Xiao *et al.*, 1999a; Xiao *et al.*, 1999b; Zhu *et al.*, 2000a). While most of our sequences were considered 'unique' in that they were different from published sequences, phylogenetic trees inferred a similar pattern to that observed where isolates from animals are used. Since identification of the zoonotic strains is still rare due to limitations in genotyping, it remains to be seen if there is any detectable variation in

zoonotic strains isolated from humans compared to those from natural animal hosts. It will also be important to investigate if there are any changes in infectivity of strains from humans back to the animal hosts.

Extensive genetic diversity within *C. parvum* and among the rest of the species within this gene locus was observed. The18S rRNA gene fragment is distinct in the *Cryptosporidium* genus, exhibiting intragenotypic variation that can reliably differentiate species and specific genotypes (Xiao *et al.*, 1999b; Xiao *et al.*, 1999a). However, our assessment on gene diversity and the presence of recombination events suggest the gene is under high selection pressure. Conversely the results suggest there has been a rapid population expansion resulting to the high number of singleton mutations especially evident in the *C. parvum* 'human' genotype group. Phylogeny based on areas under high selection tends to over-estimate the evolutionary distances between organisms. On the other hand, our target fragment was the hypervariable region that could contribute to overestimation of the distances. Yet, the locus is a common target for identification and phylogeny of *Cryptosporidium*. (Xiao *et al.*, 1999a; Xiao *et al.*, 1999b; Morgan *et al.*, 1999d; Pedraza-Diaz, *et al.,* 2001b; Guyot *et al.*, 2001; Sulaiman *et al.*, 2001; Ong *et al.*, 2002). The lack of variation based on either geographical origin or HIV status suggests 18S rRNA gene fragment may not be appropriate for typing of genetic diversity that mainly depend on environmental modifications of the gene. However, its ability to amplify different species and genotypes of the organism using one set of primers makes it appropriate for screening where the type of *Cryptosporidium* organisms occurring may be unknown.

8.0 CHAPTER 8: GENERAL DISCUSSION AND CONCLUSIONS

Intestinal parasites were prevalent in our study with over 77% of the samples having at least one parasite. This prevalence is not unusual in Kenya and other countries in the region including Somalia, Sudan and Tanzania (Kamunvi and Situbi, 1983; Chunge *et al.*, 1985; Ilardi *et al.*, 1987; Chunge *et al.*, 1991a; Magambo *et al.*, 1998). This was reassuring in the validation of our data since only one sample per participant was examined in each survey. While the results are similar to those recorded in earlier surveys, our study is, to the best of our knowledge, the first to follow the trends in the occurrence of specific intestinal parasites in non-AIDS, HIV-infected and HIV-negative people in Kenya. Other studies have reported the occurrence of intestinal parasites in HIV/AIDS patients in relation mainly to diarrhoea, where the emphasis has been on the role of opportunistic intestinal parasites among other infections (Brindle *et al*, 1993; Batchelor *et al.*, 1996; Mwachari *et al.*, 1998).

HIV was first diagnosed in Kenya in 1984 (Obel *et al.*, 1984). Since then, the incidence of the disease has steadily risen with current reports showing an estimated prevalence in adults of 14%, ranging from 5.9%-34.9% in antenatal mothers in rural areas (UNAIDS, 2001). While about 26% of our participants were HIV-infected, they were largely free of clinical AIDS. Our study therefore, gave an opportunity to profile the intestinal parasites in non-AIDS populations as they occur in a rural community setting.

We faced several limitations that affected our overall aims. Compliance was low with most participants attending only one of the three surveys. This was a major constraint for the follow-up, especially for children and HIV-infected persons who were overall, fewer in number. A previous community study in the area on knowledge, attitudes and practices (KAP) with reference to intestinal parasites, among other infections showed most people knew of the symptoms but did not rank worm infestations highly as a health concern (Kamunvi and Ferguson, 1993). While malaria, schistosomiasis and intestinal parasites are endemic in the area, the current major health concern is AIDS (Karanja *et al.*, 1997). The other limitation was that our cohort was linked to those taking part in the MTCT study that had clinical AIDS symptoms as an exclusion criterion (Songok *et al.*, 2001; MTCT study proposal, KEMRI). The MTCT study had been going on for three years prior to our survey, a factor that could have resulted in less enthusiasm for the overall project in the course of time. Time limitation within the rigid MTCT study schedules also meant mothers had only one day to attend examinations in each survey, with little room for follow-up or rescheduling. Nonetheless, we were able to carry out both cross sectional and follow-up surveys with a total of 758 stool samples examined.

Our results show there was little difference in prevalence of intestinal parasites in HIV-infected and uninfected mothers. Indeed a tendency of higher prevalence with both protozoa (except for *E. coli*) and helminths was recorded in HIV-uninfected mothers compared to the infected ones. Furthermore, HIV-infected children were no more likely to have parasites than HIV-uninfected ones except for hookworm infections where HIV-infected children had higher prevalence.

The reason for the higher hookworm infestation in children is not clear. Hookworm infections are less common in infants in endemic areas where prevalence peaks in early adolescents and remains moderately high in adults (Anderson, 1982). Hookworms are leading causes of morbidity and mortality mainly due to anaemia associated with chronic infections (Bundy and Medley, 1992; Pearson, 2002). Due to the tender age of the children in our cohort, a more stringent profile for their specific CD4+ counts would have shown the actual immune status for more precise correlation to the increased risk. This will be necessary in order to assess the progression of acute and chronic infections with hookworms among HIV-infected children where susceptibility and morbidity may be aggravated.

Our results on prevalence of intestinal parasites in relation to HIV-infection are in agreement with others done in Zimbabwe, Honduras, Ethiopia, Guinea-Bissau and Thailand (Gomez *et al.*, 1995; Lindo *et al.*, 1998; Fontanet *et al.*, 2001b; Lebbad *et al.*, 2001; Wiwanitkit, 2001). While some of these studies demonstrate, to some degree, increased enteropathy due to parasitic infections, those done on people with non-clinical HIV infection report either no apparent increase in intestinal parasites (Cegielski *et al.*, 1992; Wiwanitkit *et al.*, 2001; Lebbad *et al.*, 2001), or a decrease in prevalence (Lindo *et al.*, 1998; Anand *et al.*, 1998). The reasons for these trends are not yet clear. A study in Tanzania suggested there was an actual decrease in extracellular and lumenal parasites in HIV-positive people than HIV-negative individuals (Gomez-Morales *et al.*, 1995). Studies on the effect of HIV on the gut cells show the viral infection could induce some structural and functional impairment of the gut mucosa (Ullrich *et al.*, 1989;

Conlon *et al.*, 1990; Ullrich *et al.*, 1998; Smith *et al.*, 1999). It is proposed that the damage may selectively deter establishment of lumenal dwelling parasites (such as *T. trichiura, A. lumbricoides*, and non-invasive protozoa) (Smith *et al.*, 1988; Gomez-Morales *et al.*, 1995). Other reports suggest there may be an actual loss of helminths and extra-cellular parasites in HIV-infected persons (Lindo *et al.*, 1998). Worm expulsion can be induced by the pathological changes in the gut that result in unfavourable environment, or local inflammatory responses that result in increased permeability and exposure of the worms to components of the immune system (Garside *et al.*, 2000). Since infection and progression of HIV to AIDS results in an increase in Th2 cells activation production (a case that is also seen in helminth infection), some of which mediate parasite expulsion, it is possible there may be as yet unknown mechanisms that affect the presence and survival of specific parasite species in the gut. It is however, unlikely that such HIV-induced mucosal responses would be adequate for significant parasite clearance especially in early stages of viral infection.

Since our communities were largely free of clinical AIDS, it is possible that interaction between lumenal parasites such as *A. lumbricoides* or non-invasive protozoa, and, the human immunodeficiency virus' at this stage does not lead to any observable changes in the hosts susceptibility or establishment that would present as changes in prevalence of the former. However the high prevalence of *B. hominis* and *C. mesnili*, in both HIV-infected and infected mothers is significant. The pathogenicity of the two parasites is not yet clear with some authors suggesting the former is an opportunistic parasite (Long *et al.*, 2001; Garavelli, 2002). The high infection rates show the two parasites form a large

proportion of the enteric pathogen mix in the area. That proportion warrants further monitoring of their role in intestinal parasitic morbidity in the area.

Daily transition mechanisms of specific parasite and the relation to their overall prevalence in the community were also investigated. In a few studies where durations of infections have been determined, frequent examination of faecal samples was carried out until parasite clearance was observed (Farthing *et al.*, 1986; Gilman *et al.*, 1988; Núñez *et al.*, 1999). One of the commonly monitored parasites has been *G. lamblia* infection in children with varying durations. Using such methods Farthing and others (1986) recorded durations of 2-6 weeks among children in Guatemala. Others reported 60 days in Peru (Gilman *et al.*, 1988) and 30 days in Egypt (Sullivan *et al.*, 1988). A duration of 131.7 +/- 26.5 days was reported in Kenya for *G. lamblia* in children using Bekessy's model (Chunge *et al.*, 1991a; Chunge *et al.*, 1991b). A consequent statistical model correcting for the poor detectability for the same data using a maximum likelihood approach resulted to a reduction of the duration of infection, for *G. lamblia* to approximately 30 days each (Nagelkerke *et al.*, 1990). The resulting duration of infection was comparable to our results of 56 days in children for the same parasite.

Our results on estimates for duration of infections were robust, remaining steady in spite of low sensitivity in the diagnostic methods that would have resulted in poor parasite detectability. Where prevalence is low and stable, an increase in daily incidence of a specific parasite as would be due to an unusual increase in exposure (eg in case of waterborne parasitic outbreak), would result in a

reduction of time the average person takes to peak up the infection in the community. While the incidence would therefore increase, it may not necessarily result to a change in duration of infection. A reduction in exposure could conversely result to longer periods ‘between infections’ that might still not have much effect on the duration of infections in those already infected.

For the estimated daily clearance, a reduction in clearance rate might occur in case of a change in immunity (as may be the case in loss of maternal protection). This would results to an increase in longevity of infections and consequent increase in prevalence. Conversely, an increase in immunity against a specific parasite may lead to an increase in daily clearance that would result to a shorter duration of infection. The transition estimates, are therefore reflective to actual transmission mechanisms. The assessment of the model revealed significant findings. The adjustments and the resulting durations showed prevalence data obtained using copro-diagnostic tests such as wet-mounts or direct smears are applicable in estimating duration of infections especially for parasites with high infection rates. Such data can be used reliably to estimate the daily clearance rates of parasites in the community and relate to the prevailing prevalence. However, estimates for daily incidence and the periods ‘between infections’ may be less reliable using data obtained from low sensitivity tests.

In conclusion, our study was able to illustrate the relationships between prevalence and durations of infections in the communities studied. Numerous factors like variability in susceptibility, due to age, sex, immune heterogeneity result in different parasite prevalence in communities where exposure is similar.

For other parasites, difficulties in identification, or assumptions of 'less significance' of specific parasites by investigators leads to low reporting and therefore underestimation of prevalence. Our study highlighted the significance of application of data from parasites especially the non-pathogenic *E. coli*, as they give insights into the general parasite transmission mechanisms in the communities. Since the transitions are derivatives of several factors including those that affect incidence and cure rates, it is important to have a basic understanding of these mechanisms in order to understand factors that determine specific prevalence profiles in communities. The estimation of transition rates also gives some clues into whether high parasite prevalence for an individual parasite in different age groups is as a result of long durations or repeated infections.

In the second part of the thesis, the identification of *Cryptosporidium* parasites by genotyping was undertaken. Current knowledge on human cryptosporidiosis is largely centred on infections with *C. parvum* 'human' genotype. However, an increasing number of reports show there are more species infecting humans than previously known (Widmer *et al.*, 1998; Pieniazek *et al.*, 1999; Morgan *et al.*, 2000a; Xiao *et al.*, 2001a). Our study demonstrates the occurrence of these novel species in regions where no such reports have been documented.

In our study, *Cryptosporidium* parasites were isolated from human faecal samples of individuals living in different regions of the world. Initial analysis showed the method of preservation of such samples is crucial in subsequent DNA recovery and molecular analysis of *Cryptosporidium*. While potassium dichromate (2.5%)

offers advantages in that samples can be preserved at room temperature or at 4^0C for up to six months, we had limited success in the extraction and amplification of such samples. Our experience suggests where possible, frozen faecal samples (at -80^0) with no preservatives coupled with extractions using commercial kits offer the best results. DNA recovery did not seem to be influenced by the period samples had been frozen. The use of the commercial lysis buffer also enhanced the effectiveness of DNA recovery process from ruptured oocysts. A recent report from Thailand suggests the use of 75% ethanol as long-term copro-preservative for samples intended for *Cryptosporidium* analyses (Jongwutiwes *et al.*, 2002). This would be an appropriate alternative to potassium dichromate where immediate freezing of samples is not possible.

The 18S rRNA gene has been used extensively for the identification and characterisation of *Cryptosporidium* organisms (Morgan *et al.*, 1995; Morgan *et al.*, 2000a; Xiao *et al.*, 1999a; Xiao *et al.*, 1999b; Peng *et al.*, 2001; Pedraza-Diaz *et al.*, 2001a; Ong *et al.*, 2002). In our study, we successfully applied the hypervariable segment of this gene and identified six different *Cryptosporidium* genotypes from our study population.

Using the 18S rRNA gene fragment *C. muris* recovered from an HIV-positive patient residing in Kenya was characterised using molecular techniques for the first time (Gatei *et al.*, 2002). Positive identification of the other zoonotic species including *C. meleagridis, C. felis* and *C. canis* were made apart from the more common *C. parvum* 'human' and 'bovine' genotypes. *C. parvum* 'bovine' genotype and *C. canis* were identified for the first time in isolates from Thailand

while this was the second report to identify *C. meleagridis* and *C. felis* from this region. The results demonstrated the usefulness of the gene fragment as highly genus specific, for *Cryptosporidium*. However, we were unable to amplify two samples that were clearly positive with *Cryptosporidium* using microscopy. While the reason for this failure was not clear, it is possible that the use of a single locus is limiting where different species and strains of *Cryptosporidium* are present.

The 18S rRNA gene also exhibits heterogeneity occurring as four copies of the Type A and one copy of type B rRNA units per haploid genome (Le Blancq *et al.*, 1997; Xiao *et al.*, 1999c). In spite of the heterogeneity, we demonstrated the wide occurrence of identical subgenotypes in all geographical locations. The widest heterogeneity in the rRNA genes occurs in the first internal transcribed spacers between the 5.8S rRNA and the large subunit of the B gene with approximately 500bp more than that in the 'A' gene (Le Blancq *et al.*, 1997). There is however, only small amount of heterogeneity in the different copies of the 'A' rRNA gene, a factor that is currently used to identify the inter- and intra-species variation of *Cryptosporidium* isolates and populations (Sulaiman *et al.*, 1998; Xiao *et al.*, 1999b; Sulaiman *et al.*, 2001). Our results on nucleotide diversity suggested the high mutation rates especially in the *C. parvum* 'human' genotype may lead to overestimation of genetic distances in the species based on this locus. The results show the need to be cautious in the application of the gene fragment in fine scale phylogeny. However, the gene target remains an important tool in the precise identification of *Cryptosporidium* species especially due the wide application of a single set of primers for the different species and genotypes.

Our report and those of others illustrate the growing number of novel species infecting humans. The significance of the zoonotic species infecting human is not yet clear. Factors pertaining to the biology of the parasite and epidemiology in humans are yet to be elucidated. Different *Cryptosporidium* species have specific predilection sites of infection in the gut as seen in the natural hosts, a majority infecting the small intestine (Dubey *et al.*, 1990; Hart 1999). However, *C. muris* infects the stomach lining of infected mice while *C. meleagridis* has been isolated from the epithelia of the alveoli in turkey apart from the small intestines (Dubey *et al.*, 1990). In our case, the patient with *C. muris* infection was co-infected with *I. belli* and while the patient also presented with diarrhoea, it is not possible to speculate what role *C. muris* played in the enteric disease. There is a need to follow up the clinical pictures of patients where conclusive identification of infecting *Cryptosporidium* has have been made to clarify the resulting disease profiles.

The identification of a large proportion of zoonotic species especially in Thailand raises questions as to their origin and routes of transmission. In this study, almost half of the isolates from Thailand were of zoonotic origin. A recent report from the same region that identified *C. meleagridis, C. muris* and *C. felis* also reported that infections did not correlate with any pet ownership (Tiangtip and Jongwutiwes, 2002). It is therefore puzzling that while there may be an increased risk of cryptosporidiosis in humans due to immunosuppression, infections with *C. parvum* 'human' type that is more prevalent (in humans and possibly in their environment) appears to decrease compared to those with zoonotic species. Some

studies on human volunteers show cryptosporidiosis is more severe if it is by *C. parvum* 'bovine' genotype than 'human' genotype (Fayer *et al.*, 1998; Okhyusen *et al.*, 2001). The genotypes and strain sources, differences in infectivity and severity warrant further research in order to identify risk factors, possible transmission routes, and disease outcome in the affected people.

An isolate from a captive monkey in UK was confirmed as *C. parvum* 'human' type suggesting transmission between humans and other primates. While transmission between primates and humans are not unusual, they raise questions on the extent of host ranges of *C. parvum* 'human' genotype. There has been suggestions that *C. parvum* 'human' and bovine genotypes represent distinct species based on molecular evidence and their exclusive transmission routes. *C. parvum* 'human' genotype is considered to have an exclusive 'human to human' transmission (Fayer *et al.*, 2000). However, recent reports indicates *C. parvum* 'human' genotype can indeed infect other animals with cases of experimental infection in a ram from the south of England (Giles *et al.*, 2001) and a natural infection from a dugong (*Dugong dugon*), (Morgan *et al.*, 2000c). Hitherto, most *C. parvum*-like organisms identified in domestic animals were presumed to be *C. parvum* 'bovine' type (genotype 2). Current genotype studies show that while this genotype is widespread, others species such as *C. meleagridis* that are morphologically indistinguishable also occur in a wide host range including humans, mice and chicken, as well as the turkey (Sreter *et al.*, 2000; Xiao *et al.*, 2001b). Others like *C. felis* have been isolated in cattle (Bornay-Llinares *et al.*, 1999). There is therefore a need to clarify further the range of animal hosts susceptible to the two genotypes to support a taxonomic review.

The biggest impact of *Cryptosporidium* as a public health concern has been in the water supply and resource industry. Water supply companies in many parts of the world are struggling to eliminate *Cryptosporidium* from their supplies to the public (Fairley *et al.*, 1999). Even with the current measures, there are still too many unresolved issues regarding the public health significance of oocysts in water. The current ID50 (infectious dose) for *C. parvum* 'human' genotype in healthy volunteers varies greatly from 10-1500 (Chappell, 1998). There are no reports on ID50 levels as yet, for *C. parvum* 'bovine' genotype or other zoonotic species that are also present in water. Stringent measures in place to rid water of *Cryptosporidium* coupled with law requirements have led to a tremendous increase in the cost of water treatment. Over £8 million was required to cover monitoring costs in 121 water treatments plants in UK alone in the first year of legislation (DETR, 1998). Yet, strict monitoring may not be effective as it is not a preventive measure and consumption may have occurred. When detected, it is still not possible to predict if the oocysts are hazardous as little is known about infective doses of the zoonotic *Cryptosporidium* species. Post-treatment contamination remains a hazard despite the stringent measures (Smith *et al.*, 1989).

Data from the first part of our study confirmed that environmental contamination and transmission of parasites with a faecal-oral route is high in the rural communities. The data also suggested exposure was high in mothers and children and was similar in villages with or without piped water. While no data was

available from other regions on possible sources of zoonotic species of *Cryptosporidium,* water sources cannot be ruled out.

In developing countries, water treatment costs and monitoring are beyond the reach of many water supply authorities. Cryptosporidiosis is self-limiting in most immunocompetent persons, and induces an age-related acquired immunity in endemic regions. So far, the parasite is not ranked highly among the infectious diseases with high morbidity or mortality (Fairley *et al*., 1999b). It is therefore more feasible to carry out risk-based assessments in specific regions. This would primarily involve measures to contain overall risks of contamination in the water supply rather than targeting it as a single pathogen (Fairley *et al*., 1999a; Fairley *et al*., 1999b). Basic understanding of the different *Cryptosporidium* species infecting humans, their transmission routes and potential reservoirs should increase awareness and reduce other non-waterborne sources of infection. In the short-term, measures such as boiling water advisories, the use of bottled water or water filters for susceptible people, and general maintenance of personal hygiene standards may be more feasible in the prevention of infection with *Cryptosporidium*.

In the long term, the ubiquitous nature of the organisms, the long viability of oocysts in the environment and the wide range of genotypes that humans are susceptible to, mean that other ways of controlling the disease such as effective chemotherapy must be found. Further research into the molecular properties of *Cryptosporidium* will aid in understanding of the biology of this parasite and elucidate possible basic differences that make anti-coccidial drugs ineffective.

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Appendix 1: Ethical Considerations

Informed Consent Form

(Mothers and Children's Guardians)

Scientists at the Kenya Medical Research Institute in collaboration with your local health officers are conducting a study on parasitic infections associated with diarrhoea. Some of these infections are only being recognised recently as important infections of human beings and therefore not much scientific knowledge has been gathered previously.

We are therefore requesting you to participate in the study by allowing us to record your medical history and agreeing to give your stool sample for investigations. As you know, recovery of stool sample is a harmless procedure and will in no way have adverse effects on your condition. The results from our investigations will be given to your doctors to aid them in the diagnosis and management of any enteric diseases such as diarrhoea. Your history will aid in knowing how to handle the results that we get for proper scientific interpretation.

Your participation is purely voluntary and can be withdrawn at any time you may wish, now or during the follow-up study. You can also make further inquires about the research to the Principal Investigator, Dr Wangeci Gatei of the Centre for Microbiology Research, Kenya Medical Research Institute at Telephone number 722541 Ext. 339, 340 or through any other officer or field worker in our team. By signing the consent form we also request you to authorize the release and sharing of data from this research for scientific publications only, to agencies designated by the Principal Investigator. However personal information will remain strictly confidential and will not be disclosed whatsoever.

Declaration of Patient

I have understood the description above about the study and I sign this consent form willingly.

Signature (Client or guardian)

.... Date.............................

Patient Identification No ....

Thumb print...

Appendix 2: Trichrome stain

Protocol 1

Trichrome stain preparation-

Chromotrope 2R 6.0g, Light green SF 3.0g, Phosphotungstic acid 7.0g, Glacial acetic acid 10ml and distilled water 1000ml.

Thin faecal smears were made on clean glass slides and dried in a 37^0C incubator overnight.

Smears were then fixed in 70% ethyl alcohol for 2 minutes.

They were placed in undiluted trichrome stain for 10 min.

The stain was then drained and the excess blotted on paper.

Smears were dipped briefly in 99% acid alcohol (1% HCl, 99% Ethanol)

The smears were then quenched in 100% ethanol for 30 sec and then two into two changes of ethanol at

a) 100% ethanol for 5 min
b) 100% ethanol for 5 min

The slides were then placed in two changes Xylene for 10 min

They were examined at 400X confirming at 1000X under oil.

The stain is useful for a wide range of intestinal protozoa especially trophozoites. Cytoplasm of organisms stain blue/green tinged with purple while nuclear chromatin, erythrocytes and bacteria appear purplish/red. Yeast, moulds and debris stain green or red.

For oocyst forming protozoa, Ziehl Neelsen acid-fast stain was used as described in the second part of the thesis. This was done for samples preserved in potassium dichromate.

Appendix 3: Transition Estimates for daily incidence and clearance rates

Only participants seen in each consecutive examination are included in the estimates. The model is based on two proportions:

	Exam 2		
	-	+	
Exam 1 -	A	B	A + B
+	C	D	C + D
	A+C	B+D	A+B+C+D

α = Proportion of parasite positive people at the second survey, in those negative at the first

β = Proportion of parasite negative people at the second survey in those positive at the first

$$\alpha = B/(A+B)$$

$$\beta = C/(C+D)$$

t = Time in days between examinations

Formulae 1

$$\hat{h} = \frac{\alpha}{t(\alpha+\beta)} \ln \frac{1}{1-(\alpha+\beta)}$$

$$\check{r} = \frac{\beta}{t(\alpha+\beta)} \ln \frac{1}{1-(\alpha+\beta)}$$

$\hat{h}$ = daily incidence; $1/\hat{h}$ = estimated duration of parasite-free period

$\check{r}$ = daily clearance rate $1/\check{r}$ = estimated duration of infection

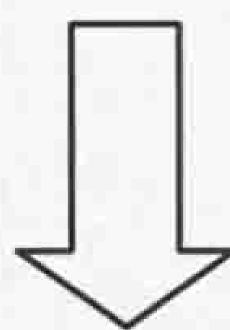
Evaluation of Diagnostic tests in the application of Bekessy's model

Adjustment was worked out based on an assumed proportion of error in parasite detectability. A constant (k) was assumed to be that proportion of the negatives that were really negatives in the first and second examinations. Applying that adjustment therefore, it was assumed that if 1/10 samples were false negatives (that is one tenth of the total negatives were actually positives) then k is 9/10 = 0.9.

The above table used in the number of participants positive or negative in the first and second examination was therefore worked out as follows:

Initial Counts

		Exam 2		
		-	+	
Exam 1	-	A	B	A + B
	+	C	D	C + D
		A+C	B+D	A+B+C+D



Corrected Counts

		Exam 2		
		-	+	
Exam1	-	k^2 (A)	B	k (A + B)
	+	C	D	C + D
		k (A+C)	B+D	A+B+C+D

Where k was formulated as follows

The total number of participants in the initial and ‘corrected’ observations remained the same (A+B+C+D). The change therefore was in the number of observations identified as negative or positive. In cell ‘A’ observations were identified as negative in the first and second examination. To make the adjustment it was assumed that a ‘k’ proportion of the observations in ‘A’ were actually negative. This number included those identified in the first examination and second examinations as negative.

A cell therefore represents total of negatives observed twice. Applying (k) the proportion of counts presumed true negatives in the first and second examination (two examinations):

Total negatives in the first and second examination is therefore $k^2(A)$

Adjusting for total negatives in the first examination (one examination adjustment)

$k(A+B)$

Adjusting for total negatives in the second examination (one examination adjustment)

$k(A+C)$

The resulting counts were thereafter applied in the daily transition rates and thereafter the estimated durations of infections and the durations of parasite free periods as before.

Appendix 4: Modified Ziehl Neelsen (ZN) Staining (Ma and Soave, 1984)

Thin smears of 2.5% potassium dichromate preserved faecal samples were prepared and dried for at least 1 hour on pre-cleaned frosted microscopic glass slides.
The samples were then fixed in 100% methanol for 5 minutes and stained using the modified ZN acid fast stain as follows: Slides were

1. Immersed in cold concentrated carbol fuchsin solution (BDH Laboratory Supplies, England) and stained for 15 min.
2. Smears were then rinsed in tap water and decolourised with 1% acid alcohol and briefly rinsed in tap water
3. They were counterstained with 0.4% malachite green (BDH Laboratory Supplies, England) for 30 sec, rinsed in tap water and air dried

Slides were then scanned at X400 and confirmed in oil immersion at X1000 magnification.

Appendix 5: Summary of sequences analysed

All frequencies are given in percent (without primer regions)
Sequence from GenBank included for comparison

Nucleotide Frequencies

C. meleagridis

	T	C	A	G	Total	
K53	30.5	15.1	33.6	20.7	776	Ø
Th19	30.5	15.1	33.6	20.7	776	Ø
Th22	30.6	14.9	33.5	21.0	778	
Th15	30.5	14.8	33.8	20.9	776	
Th1	30.5	14.9	33.8	20.7	776	
Th27	30.7	14.8	33.6	20.9	776	
*Cmel	30.7	14.8	33.8	20.7	776	
Th3	30.4	14.9	33.9	20.7	776	

C. parvum 'human' genotype

	T	C	A	G	Total	
Vt13	30.8	14.8	33.2	21.2	777	Þ
Th20	30.8	14.8	33.2	21.2	777	Þ
M15	30.5	15.1	33.3	21.1	777	
Th18	30.5	14.9	33.3	21.2	777	
K52	31.1	14.6	33.1	21.2	779	
k27	30.9	14.7	33.1	21.4	777	
M10	31.0	14.9	33.1	21.0	780	
M1	31.2	14.6	33.1	21.2	780	
Th30	30.9	14.8	33.1	21.2	779	
M11	30.9	14.9	33.2	21.0	780	

****

C. parvum 'human' continued					
K24	30.9	14.7	33.3	21.1	777
Th28	31.0	14.7	33.4	21.0	778
*Cprm1	31.1	14.7	33.3	21.0	778
UKM	30.9	14.9	33.3	20.9	780
M6	30.9	14.8	33.4	20.9	779
K25	31.2	14.6	33.3	20.9	780 §
Th31	31.2	14.6	33.3	20.9	780 §
Th32	31.0	15.0	33.1	20.9	780
Th35	31.0	14.7	33.3	21.1	778
K32	31.2	14.6	33.3	20.9	780 §
Th33	31.2	14.6	33.3	20.9	780 §
*Cph7-1	31.2	14.6	33.3	20.9	780 §
K28	31.2	14.6	33.4	20.8	779
UKL4.	31.2	14.6	33.3	20.9	780 §
Bz16	31.2	14.6	33.3	20.9	780 §
Bz18.	30.7	14.7	33.3	21.2	774
K46	31.0	14.7	33.2	21.0	780
*Hcnv4	31.2	14.6	33.3	20.9	780 §
Bz12	31.2	14.6	33.3	20.9	780 §
M8	31.2	14.6	33.5	20.8	780
Th24	31.0	14.9	33.1	21.0	780
C. parvum 'dog' genotype (*C. canis*)					
Th34	31.0	14.8	33.3	21.0	772 ¥
Cpd1	31.0	14.8	33.3	21.0	772 ¥
C. parvum 'cat' genotype (*C. felis*)					
Th26	32.3	14.6	32.6	20.4	807
Th7	32.3	14.7	32.3	20.6	807
*Cat1	32.6	14.5	32.5	20.4	807
C. parvum 'monkey' genotype					
*Cpm1	30.9	14.7	33.8	20.6	781

C. parvum 'bovine' genotype

	T	C	A	G	Total	
Th2	31.0	14.7	33.3	20.9	780	
*Cpbksu-1	30.5	15.0	33.6	20.9	774	
*Cpboh-6	30.5	14.8	33.7	21.0	777	¢
*Cpksu-1	30.5	14.8	33.7	21.0	777	¢
K29	30.5	14.8	33.7	21.0	777	¢
M14	30.5	14.8	33.7	21.0	777	¢
Th13	30.8	14.8	33.5	21.0	777	
K39	30.6	14.9	32.8	21.7	774	
K4	30.5	15.0	33.6	20.9	780	
C. muris						
K33	28.2	17.4	31.3	23.1	776	#
*Cmidrh	28.2	17.4	31.3	23.1	776	#
Total Avg.	**30.8**	**14.9**	**33.3**	**21.0**	**779.4**	

Ø Denotes sequence identical but not with published sequences

Þ Denotes sequence identical but not with published sequences

* Denotes sequence retrieved from the GenBank.

§ Denotes sequence has 100% identity with published HCNV4 and CPH7

C. parvum human genotype

¢ Denotes sequence has 100% identity to published CPBOH and KSU

C. parvum bovine genotype

¥ Denotes sequence has 100% identity with published CPD1 *C. parvum* 'dog' genotype

# Denotes sequence has 100% identity with published *C. muris*

All other unmarked sequences did not show 100% sequence identity with any published sequences at this locus and were considered 'unique' strains' (Blast Search in Feb 2002)

Cryptosporidium like other Apicomplexa is AT-rich which comprised over 64% of the nucleotides.

Cryptosporidium muris Infection in an HIV-Infected Adult, Kenya

Wangeci Gatei,*†Richard W. Ashford,* Nicholas J. Beeching,* S. Kang'ethe Kamwati,‡ Julie Greensill,† and C. Anthony Hart†

We describe a case of *Cryptosporidium muris* infection in an HIV-infected adult with diarrhea in Kenya. Sequence analysis of an 840-bp region of the 18S rRNA gene locus demonstrated the isolate had 100% nucleotide identity with *C. muris* recovered from a rock hyrax, 98.8% with a *C. muris* "calf" isolate, 95.5% with *C. serpentis*, but only 87.8% with *C. parvum* "human" type.

Tyzzer identified the first *Cryptosporidium* species, *C. muris*, in the gastric glands of mice (1). Thereafter, he identified *C. parvum*, which infects the small intestines of many mammals, and described the complete coccidian life cycle. Over the next 70 years, more than 23 different species of the genus were described on the basis of their morphology and natural hosts. However, when animals were experimentally infected, many of the described *Cryptosporidium* species were found to be identical. In the 1970s the classification was revised, and today only six to eight species are recognized as valid, with most human, zoonotic, and mammalian infections being attributed to the different *C. parvum* genotypes (2,3). *C. muris*, which is naturally a murine parasite, appears to have a more limited host range than *C. parvum*. Experimental transmission studies of *C. muris* have shown that the isolate from laboratory mice can infect other animals, including dogs, guinea pigs, rabbits, lambs, and gerbils, although it did not produce patent infections (4). The parasite has also been isolated from a rock hyrax (*Procavia* sp.) from a zoo and a Bactrian camel with chronic cryptosporidiosis (3,5). For many years, the parasite was thought to infect cattle; however, recent studies have shown that the *C. muris* that infects cattle is genetically distinct, and a new species name, *C. andersoni*, has been suggested (6).

Conventional diagnostic methods for *Cryptosporidium* do not differentiate the various species and genotypes, and most infections are diagnosed as *C. parvum*. *C. parvum* "human" and "bovine" genotypes remain the main causes of human cryptosporidiosis, but lately identification of infections with other genotypes and also *Cryptosporidium* species other than *C. parvum* has increased in both immunosuppressed and immunocompetent persons (7-10). Possible asymptomatic human infection with *C. muris* was reported in two healthy girls in Indonesia (11). Morphologic studies on the oocysts showed they were most likely to be *C. muris,* although there was no genotypic or experimental animal confirmation. Phylogenetic analysis has enabled more conclusive assignment to species and genotypes infecting humans and other animals (12,13). We report a case of *C. muris* infection, confirmed by morphology and genotyping, in an adult HIV-infected man from Kenya, hospitalized with diarrhea.

The Study

Fecal samples were collected from diarrheal patients from a hospital in Nairobi, Kenya, as part of a larger study. The patient described was an HIV-infected man who had clinical tuberculosis and diarrhea. *Isospora belli* was also detected in a fecal sample from the patient.

The fecal samples were preserved in both sodium acetate formalin and 2.5% potassium dichromate and kept at 4°C. They were stained with Kinyoun's carbol fuchsin modified acid-fast stain and examined by oil immersion microscopy. An aliquot of 400 μL of the sample suspension in 2.5% potassium dichromate was processed for genotypic analysis. Potassium dichromate was washed 5 times with cold, distilled water until the yellow color cleared. Oocysts were ruptured by freeze-thaw, and DNA was extracted by using a QIAamp DNA Mini Kit (Qiagen, West Sussex, UK) for stool DNA purification as per protocol.

A section of the SSU (18S) rRNA gene was amplified by nested polymerase chain reaction (PCR) as described (14), using the forward primers 5'-TTCTAGAGCTAATACATGCG-3' and the reverse primer 5'-CCCTAATCCTTCGAAACAGGA-3' for primary PCR. Secondary PCR used primers 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and reverse primer 5'-AAGGAGTAAGGAACAACCTCCA-3', employing the Techne (FTGENE2D Techne, Cambridge Ltd., UK) thermal cycler. Restriction fragment analysis of the secondary PCR product was done by digesting 15 μL of product in a 40-mL total reaction volume consisting of 15 U of *Ssp*1 and 3 μL of restriction buffer (Boehringer Mannheim Biochemicals, Indianapolis, IN) for species identification and *Asn*1 (Boehringer Mannheim) for genotyping in the same concentration at 37°C for 1 hour. Digestion products were separated on 2% agarose gel and visualized by ethidium bromide staining. The internal (secondary) fragment was purified by using the Prep-A-Gene DNA purification kit and cloned into PGEM-T Easy plasmid vector (Promega Corporation, Madison, WI) as described by the manufacturer. The cloned product was sequenced and aligned with previously published sequences of the 18S rRNA gene of *Cryptosporidium* species by using the CLUSTALX (EMBL, Heidelberg, Germany) program and manual adjustments. Multiple alignment was done with the Phylogeny Inference Package (PHYLIP version 3.5c, J. Felsenstein and the University of Washington, Seattle, WA). Sequences were analyzed by using

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DNADIST followed by neighbor joining (NEIGHBOR, PHYLIP package). One hundred replica samplings were analyzed for percentage bootstrap values. Accession numbers for *Cryptosporidium* 18S rRNA genes used were AF093498, AF093497, AF093496, AF108866 and AF093489, AF093499, AF112569 AF115377.

Results

Microscopic examination of the acid-fast stained fecal smear revealed ovoid oocysts that were an average size of 7.5-9.8 x 5.5-7.0 µm (Figure 1). Cysts of *I. belli* were also identified in the stained smear.

Restriction endonuclease digestion with *Ssp*1 of the secondary PCR 18S rRNA product yielded two fragments of 385 bp and 448 bp in size, while *Asn*1 digestion yielded two visible bands that were 102 bp and 731 bp. The results match restriction fragment patterns observed following similar digestions of *C. muris* amplicons from rock hyrax and Bactrian camel isolates (14).

The resulting sequence 18S r RNA gene fragment of the *C. muris* human isolate was deposited in the EMBL Nucleotide Sequence Database (Accession no. AJ307669). Sequence analysis with ClustalX showed this human *C. muris* isolate had a 100% nucleotide identity to that of a *C. muris* isolate from a rock hyrax and a Bactrian camel (EMBL Accession no. AF093498, AF093497), 98.8% identity to a *C. muris* "calf" isolate (AF093496), 96.5% with *C. serpentis* (AF108866), and only 87.8% identity to *C. parvum* human type (AF093489). *C. muris* calf isolate (AF093496) has since been shown to be a different species from *C. muris* ("mouse" type, Accession no. AF093498) and has been given a new name, *C. andersoni*. The phylogenetic tree showed topology similar to that already published for *Cryptosporidium,* with *C. parvum* clustering in one clade, and our patient's sample and published sequences of *C. muris* (rock hyrax isolate)*, C. andersoni* (calf isolate), and *C. serpentis* clustered in another group (Figure 2).

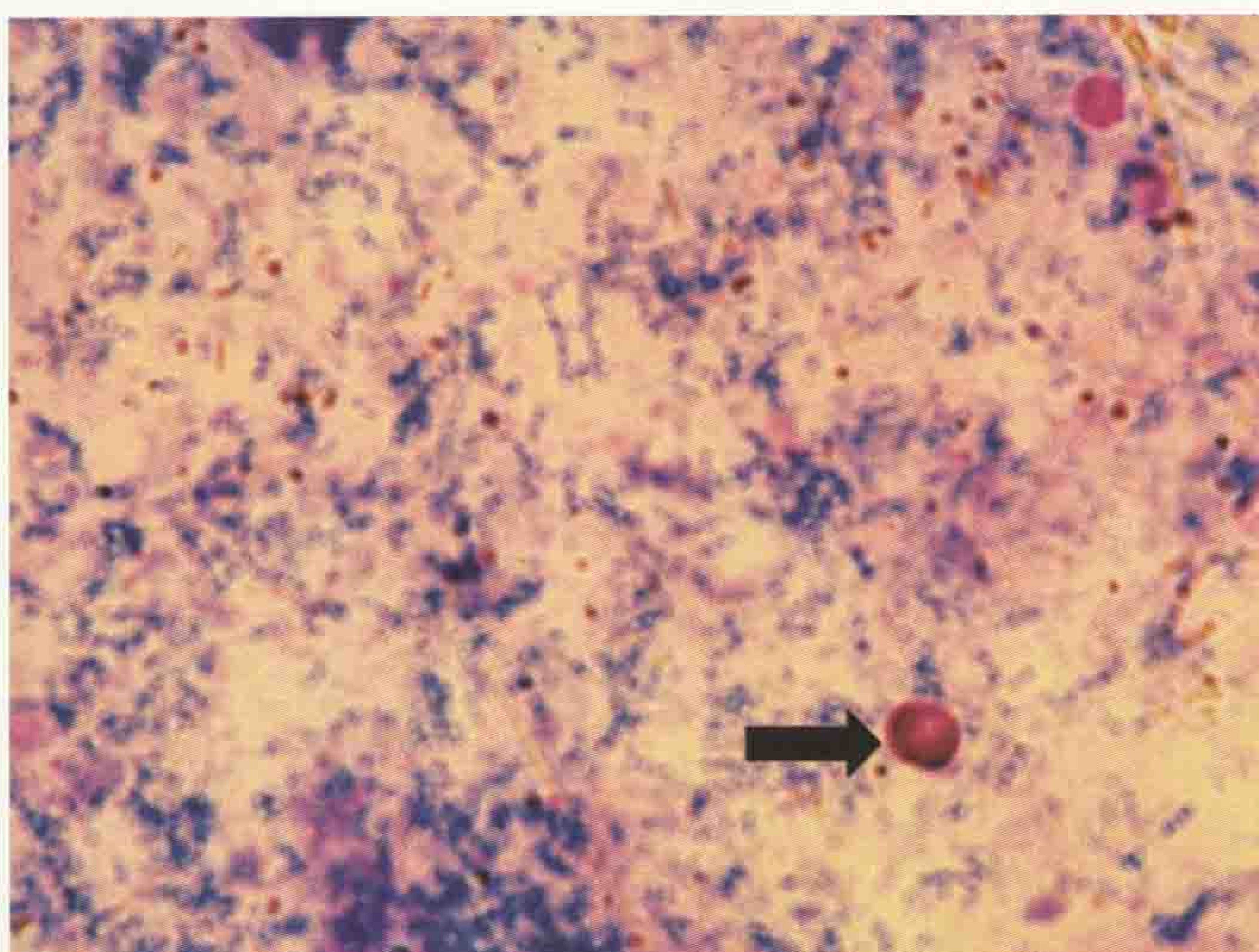

Figure 1. *Cryptosporidium muris* oocysts (under oil X 1,000), stained by Kinyoun's acid-fast staining.

Conclusions

Our study used genotypic analysis to confirm microscopic detection of *Cryptosporidium* oocysts in fecal samples and indicated that *C. muris* can indeed infect humans. Although immunosuppression has been observed to produce an increased susceptibility to cryptosporidiosis, the range of *Cryptosporidia* that can cause human cryptosporidiosis is still being elucidated (8,13,15). Lately, novel genotypes and *non-C. parvum* species such as *C. meleagridis*, *C. felis,* and *C. parvum* "dog" type have been identified not only in HIV-infected persons but also in HIV-uninfected patients (7,9,10). Genotypic analysis of *Cryptosporidium* organisms in fecal samples in the United Kingdom showed the occurrence of *C. meleagridis, C. felis,* and *C. parvum* "dog" type in immunocompetent and immunosuppressed persons (10,16). In another study in Peru, *C. felis, C. parvum* "dog" type, and *C. meleagridis* were identified in children not infected with HIV. In that study, *C. meleagridis* was as common as *C. parvum* "bovine" type; it appeared to be a stable part of the enteric pathogen mix causing cryptosporidiosis, perhaps only being identified with current definitive molecular methods (9).

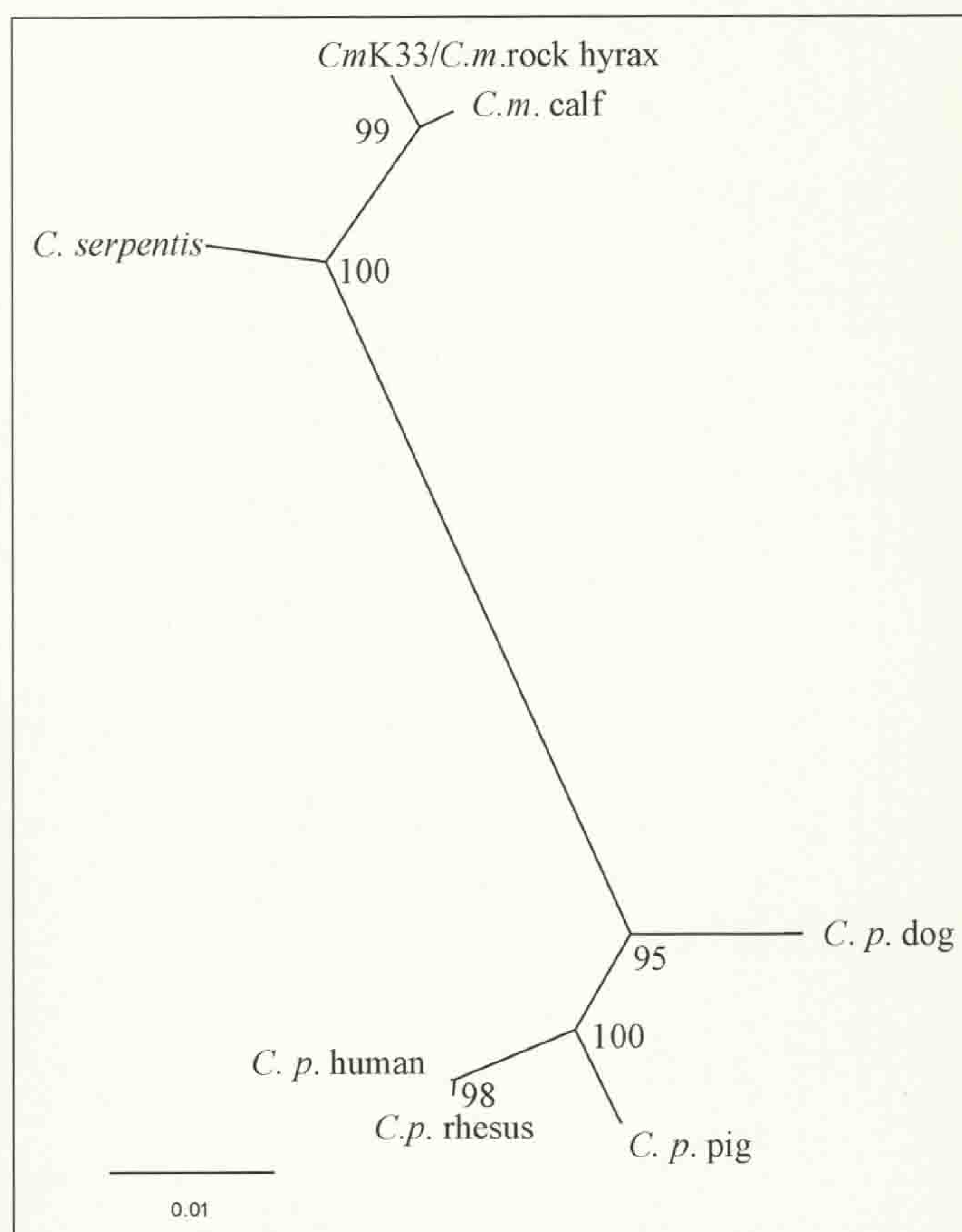

Figure 2. Comparison of 18S rRNA gene sequences of *Cryptosporidium* species. K33-*C.muris* from human patient (current paper Accession no. AJ307669); *C. m (muris)* "rock hyrax" (Accession no. AF093498); *C.m. (muris)* "calf" isolate (AF093496), now renamed *C. andersoni; C. serpentis* (AF093499); *C.p (parvum)* "dog" (AF112576); *C.p.* "rhesus monkey" (AF112569); *C.p.* "human" (AF093489); and *C.p.* "pig" (AF115377). Numbers refer to the percentage of repeated analyses that gave the same tree topology (bootstrap values).

C. muris infects the gastric glands of immunocompetent or nmunocompromised (nude and SCID) mice (17); however, nce our patient was co-infected with *I. belli*, the role of *C. uris* in our patient's gastroenteritis and its possible site of nfection in this patient are unclear.

A report of possible asymptomatic *C. muris* infection in ealthy persons (11) and our finding of it in an immunosupressed patient suggest that this may be yet another *ryptosporidium* species with a zoonotic potential. The range f animal reservoir hosts in which *C. muris* has been identified r experimentally transmitted adds to the importance of *ryptosporidium* species as a public health concern (3,4,15). he current genotypic analyses are making it possible to make nore conclusive diagnoses and to speculate on possible ources of infection (14-16). These techniques will need to be pplied more widely to identify and characterize isolates of *ryptosporidium* for more definitive epidemiologic mapping.

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Wangeci Gatei is a Commonwealth Universities Scholar, working oward a doctorate in parasitology in the Tropical and Medical chools, University of Liverpool. She qualified in veterinary medicine in 1987 and since then has worked at the Kenya Medical esearch Institute in Nairobi as a veterinary parasitologist.

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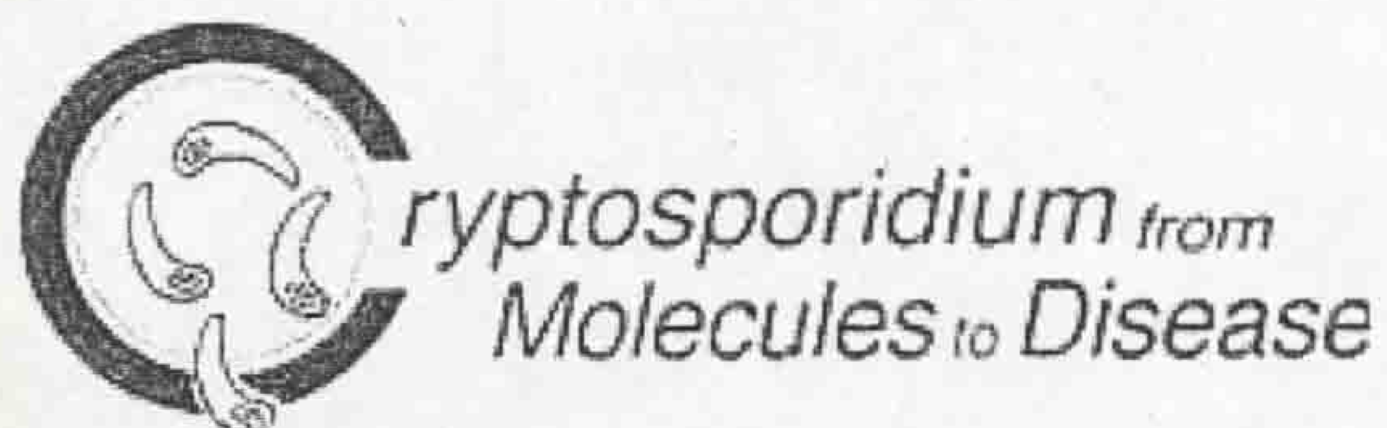

Cryptosporidium from Molecules to Disease

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GENOTYPIC VARIATION OF *CRYPTOSPORIDIUM* PARASITE RECOVERED FROM PERSONS LIVING IN KENYA, MALAWI, VIETNAM AND BRAZIL

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Cryptosporidium organisms were isolated from fecal samples taken from children and HIV positive and negative adults living in Kenya Malawi, Vietnam and Brazil. A section of 18S rRNA gene from the isolates was amplified by nested PCR followed by restriction fragment length polymorphism and sequencing. Multiple alignment with published sequences and phylogenetic trees were used to determine the sample sequences and genetic diversity. Initial results indicated the prevalence of *C. parvum* human and bovine genotype, varying among countries, age groups and HIV status. So far over 70% of the samples from Kenya and Malawi showed they were *C. parvum* human genotype with the rest being *C. parvum* 'bovine' genotype. A case of *C. meleagridis* and *C. muris* were detected in HIV infected persons from Kenya. Analysis is continuing and full results will be presented. So far the results corroborate what is currently being found by others showing diverse species and genotypes of *Cryptosporidium* occur together in endemic areas.

Key words- *Cryptosporidium*, 18s rRNA gene, HIV

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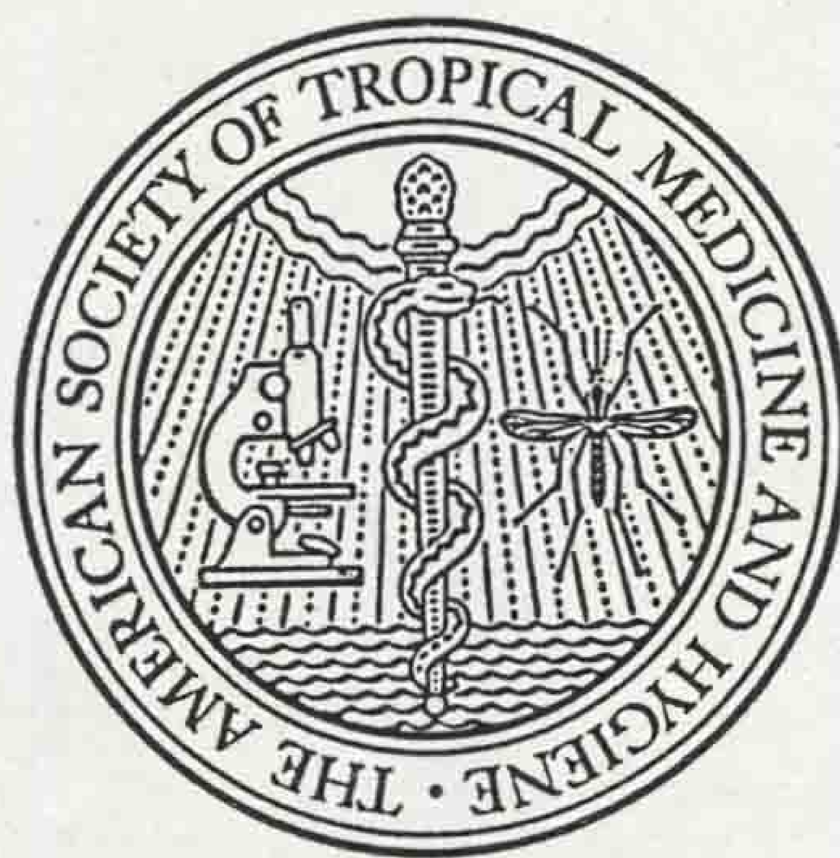

Abstract No. 1417

Genotypic analysis of *Cryptosporidium* parasites from persons with or without HIV living in Kenya, Malawi, and Vietnam.

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Cryptosporidiosis is a common infection in children and immunosuppressed persons. Recent genotypic analysis of isolates recovered from both immunocompetent and immunocompromised persons suggest that apart from the established *C. parvum* human parasites, other genotypes and different species of the organism may be causing cryptosporidiosis but their role in the epidemiology of the disease is largely unclear due to limitations in the current routine diagnostic methods. We examined the genotypic characteristics of *Cryptosporidium* isolates on the polymorphic section of the small subunit (SSU) rRNA gene locus, recovered from both HIV negative and HIV positive persons living in Kenya, Malawi, Brazil and Vietnam. Analysis was done by multiple alignment of the partial sequences and phylogenetic trees were used to identify and determine their genetic relationships with the already published sequences. Our results demonstrate the occurrence *C. parvum* 'human' and 'bovine' genotypes, but also the presence of other species including *C. meleagridis* and *C. muris* confirming the heterogeneity of the genus that cause human cryptosporidioisis.

"Monological thinking looks for single answers – and there are none."

Buckminster Fuller