CRYPTOSPORIDIOSIS IN JEDDAH CITY SAUDI ARABIA

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

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ABSTRACT

The study has three components. The first component is a prospective cross sectional study undertaken between October 2001 and April 2002 in Jeddah city, Saudi Arabia to detect the prevalence and seasonality of *Cryptosporidium* spp infection in children aged 0-6 years and to define the factors which may predispose to risk of infection in this age group. The study involved examination of faecal samples from 230 diarrhoeal cases and 322 non-diarrhoeal controls. The samples were microscopically examined with iodine, Ziehl Neelsen, and Auramin Phenol staining methods. *Cryptosporidium* antigen was detected in the faecal samples by using the Alexon prospect *Cryptosporidium* ELISA kit. The overall prevalence of *Cryptosporidium* infection was 9.6 % in the cases and 0.6 % in the controls, OR= 16.92, 95% CI = 3.8-105 and $P<0.001$). The seasonality of the infection significantly varied between winter 20.8 % and spring 79.2 % (OR= 4.21, 95% CI = 1.43-13.27 and $P<0.001$). Contact with other persons with history of diarrhoea was the only factor significantly increased the risk of the infection ($P=0.05$). Other parasitic infection were identified in both cases and controls namely *Cyclospora* spp (3 %, 0 %), *Entamoeba histolytica/dispar* (11 %, 5 %), *Giardia lamblia* (9 %, 3.7 %), *Blastocystis hominis* (0.9 %, 1.6 %), *Ascaris lumbricoides* 1.7 %, 2.5 %), *Entrobious vermicularis* (0.9 %, 2.8 %), Hookworm (0 %, 0.6 %), and *Trichuris trichuria* (0.9 %, 0.3 %).

In the second part of the study, 11 more *Cryptosporidium* positive samples were collected in a second visit to the area between December 2002 and January 2003. *Cryptosporidium* species were identified by targeting the hyper-variable region of the 18S rRNA gene and part of the first domain (N terminal) of the COWP gene. Four different species were found, *C.hominis* 37 %, *C.parvum* 42.9 %, *C.meleagridis* 2.9 %, and *C.muris* 2.9 %. One isolate exhibited a mixed infection with *C.parvum* and *C.hominis*. The majority of the isolates (63.2 %) collected in the spring were zoonotic in origin. Nucleotide diversity was most extensive in *C.parvum* that had higher singleton mutation rates 7.7 per gene locus compared to *C.hominis* which had a singleton mutation rate of 6.7 and 0.75 in 18S rRNA and COWP gene respectively. The sub-genotypic identification of *C.parvum* and *C.hominis* isolates was done by targeting the sporozoite antigen gp15/45/60 gene. A total of 5 different allelic groups were determined in *C.hominis* (Ia, Ib, Id) and *C.parvum* (IIa , Ic).

Finally, 130 serum samples from adults (18-30) years in the same city were examined to identify the sero-prevalence of cryptosporidiosis infection and to determine the factors associated with increase risk of infection. Western Blot analysis (WB) with two different antigen markers were used, the 15-17 KDa and the 27 KDa. Among the participants, 8.5 % had antibodies to the 15 KDa, 23.8 % had antibodies to the 27 kDa, a 34.6 % were sero-positive to both antigens, and 33.1 % were sero-negative to both antigen markers. Source of drinking water with a strong association with drinking tap water was the only factor significantly associated with seropositivity to cryptosporidiosis infection (OR= 37.33, $P< 0.001$).

DEDICATION

TO MY MUM AND AUNT FOR THEIR LOVE AND PATIENCE

TO MY HUSBAND FOR HIS LOVE AND SUPPORT

TO SALMAN AND FAI FOR SACRIFYING

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LIST OF ABBREVIATIONS

Ab	Antibodies
AP	Auramin Phenol
BDH	Bund Deutscher Heilpraktiker ®
Blast	blast local aligment search tool
BSA	Bovine Serum Alboumine
$CD4^+$ T	Cluster of Differentiation 4 T lymphocyte
$CD8^+$ T	Cluster of Differentiation 8 T lymphocyte
CDC	Centers of Disease Control and Prevention
CHEF	Countour-Clamped Homogeneous Electrophoresis Field
95%CI	95% Confidence intervals
CNS	Central Nervous system
COWP	*Cryptosporidium* Oocysts Wall Protein
CSA	*Cryptosporidium* specific antigen
dhfr-ts	dihydrofolate reductase-thymidylate synthase gene
DNA	Dieoxy ribonucleic Acid
dNTP	deoxynucleotide triphosphate
ds-RNA	double-stranded Ribonucleic Acid
EDTA	Ethylenediamine Tetraacetate
ELISA	Enzyme Linked Immunosorbent Assays
elF-4A	Initiotion Translation Factor
gp/15/45/60	Glycoprotein sporozoites antigen 60 KDa gene
GPI	Glycophosphatidyl Inositol
HAART	Highly Active Antiretroviral Therapy
HCl	Hydrochloric acid
HIV/AIDS	Human Immune-Deficient Virus/ Acquired Immune-Deficiency Syndrome
HPLC	High Performance liquid Chromatography
HSP 70	Heat Shock Protein 70 Kilodalton gene
ID_{50}	Infective dose
IFN-γ	Interferone- γ
IFAT	Immunofluorescence Antibody Test
IgA	Immunoglobulin class A
IgG	Immunoglobulin class G
IgE	Immunoglobulin class E
IgM	Immunoglobulin class M
IL-12	Interlukine-12
IPTG	Isopropyl-β-D-galactopyranoside
ITS-1	Internal Transcribed Spacer 1
ITS-2	Internal Transcribed Spacer 2
IVDUs	Intravenous Drug Users
KFMR	King Fahad Medical Research Centre
LB	Luria broth
LSU	Large sub unite gene
LSTM	Liverpool School of Tropical Medicine
MCK	Modified Cold Kinyoun
MgCl	Magnesium Chloride

ML	Maximun Likelihood
MP	Maximum Parsimony
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NJ	Neighbour-joining
OD	Optical Density
OR	Odds ratio
P	Probability value
PCR	Polymerase Chain Reaction
PE	Perkin Elmer
pGEM-T	Vector system (Promega)
PHLS	Public Health Laboratory Service
poly (T)	Polytheronine locus
PV	Parasitophorus Vacuole
PVM	Parasitophorous Vacuolar Membrane
RAPD	Random Amplified Polymorphic
rRNA	Ribosomal Ribonucliec Acid
RFLP	Restriction Fragment Length Polymorphism
RNR	Ribonucleotide Reductase
*Rsa*I	*Rhodopseudomonas sphaeroides* derived endonuclease
SAAPs	Single Amino Acid Polymorphisms
SDS	Sodium Dodecyl Sulphate
SNPS	Single Nucleotide Polymorphism
SSCP	Single-Strand Confirmation Polymorphism
*Ssp*I	*Sphaerotilus* derived endonuclease
SSU rRNA	Small Subunit Ribosomal Ribonucleic Acid
Taq	*Thermus aquaticus* derived polymerase
TBE	Tris Borate EDTA
TMB	Tetrametyl-Benzadine Buffer
TNF-α	Tumour Necrosis Factor
TRAP-C1	Thromp-spondine related adhesion protein 1 *Cryptosporidium* gene
TRAP-C2	Thromp-spondine related adhesion protein 2 *Cryptosporidium* gene
UK	United Kingdom
USA	United States
*Vsp*I	*Arthrobacter* derived endonuclease
WB	Western Blot
WHO	World Health Organization
w/v	weight by volume
X^2	Chi square value
ZN	Ziehl Neelsen

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CHAPTER ONE

INTRODUCTION

Cryptosporidiosis is an acute diarrhoeal disease caused by *Cryptosporidium* spp. *Cryptosporidium* is a genus of protozoan parasite belonging to the phylum Apicomplexa. Members of this genus are causative agents of intestinal, gastric, or respiratory cryptosporidiosis in a range of animal and/or human hosts, particularly young individuals (O'Donoghue, 1995; Fayer *et al.*, 2000). *Cryptosporidium* continues to emerge as a significant enteric pathogen in immuno-compromised patients as well as immuno-competent hosts (Current & Garcia, 1991). Infections are not uncommon in travellers and those working or living in agricultural environments and in children in day-care settings (Miron *et al.*, 1991; Tangerman *et al.*, 1991; Lengerich *et al.*, 1993).

Infected individuals show a wide spectrum of clinical presentations, but the pathogenicity of *Cryptosporidium* varies with the species of parasites involved and the type, age, and immune status of the host (Xiao *et al.*, 2004). The clinical signs of cryptosporidiosis include diarrhoea and associated dehydration and weight loss, colic, nausea, malabsorption and wasting; however, in immuno-competent people, the disease is usually self-limiting (DuPont *et al.*, 1995). Perhaps the greatest impact of *Cryptosporidium* infection is in people with impaired immunity from any cause. This includes patients on immunosuppressive therapy, transplant patients, patients with congenital immune defects especially in cellular immunity and most importantly Human Immuno-deficiency Virus/Acquired Immuno-Deficiency Syndrome (HIV/AIDS) patients. In HIV/AIDS patients it normally presents as chronic unremitting diarrhoea and occasionally involves other systems of the body a part from the gastrointestinal tract (McGowan *et al.*, 1993). Cryptosporidiosis is often severe and can be fatal in this population (Clark, 1999).

Cryptosporidiosis is now considered to be an important cause of acute childhood diarrhoea in both developed and developing countries but more so in the latter

(Guerrant, 1997; Clark, 1999). It is also an important cause of persistent diarrhoea in children especially in developing countries and contributes significantly to childhood malnutrition and poor weight gain (Molbak *et al.*, 1993; Checkley *et al.*, 1997).

In developing countries *Cryptosporidium* infections occur mostly in children younger than five years, with a peak occurrence of infections and diarrhoea in children younger than two years (Bhattacharya *et al.*, 1997; Bern *et al.*, 2000). The prevalence of *Cryptosporidium* infection in children in various studies ranges from 2%-40.7% and among HIV/AIDS patients from 14%-50% with developing countries having the higher prevalence (Clark, 1999; Newman *et al.*, 1999). Sero-prevalence studies show much higher rates ranging from 25-35% to 42-57% in developed and developing countries respectively (O'Donoghue, 1995). However, children in general can have multiple episodes of cryptosporidiosis, indicating that immunity acquired against *Cryptosporidium* infection is short lived or incomplete or there are numerous different strains that do not give cross-protection (Newman *et al.*, 1999).

Because *Cryptosporidium* spp infect humans and a wide variety of animals and because of the ubiquitous presence of *Cryptosporidium* oocysts in the environment, humans can acquire *Cryptosporidium* infections through several transmission routes (Clark, 1999). Transmission occurs through the faecal–oral route in animal-to-human or human-to-human contact, by recreational exposure to contaminated water or land, or by consumption of contaminated water and food (Smerdon *et al.*, 2003). In paediatric and elderly populations, especially in day care centres and nursing homes, person-to-person transmission probably plays a major role in the spread of *Cryptosporidium* infections (Neill *et al.*, 1996). In rural areas, zoonotic infections via direct contact with farm animals have been reported many times, but the relative importance of direct zoonotic transmission of cryptosporidiosis is not entirely clear (Miron *et al.*, 1991). In industrialized countries, epidemic cryptosporidiosis can occur in adults by the food-borne or waterborne route (Millard *et al.*, 1994; Quiroz *et al.*, 2000). Numerous outbreaks of cryptosporidiosis due to contaminated food or water, by drinking or recreational activities have been reported and studies have

pointed out water as the major route of transmission of *Cryptosporidium* (Gallaher *et al.*, 1989; MacKenzie *et al.*, 1995).

Detection depends on observing the presence of intact oocysts in faeces. The earliest method of staining of a faecal smear by the Ziehl Neelsen (ZN) (Modified Acid Fast) method remains the quickest and the easiest to perform. Other methods used in clinical diagnostic laboratories include direct or indirect immunofluorescence Antibody Test (IFAT) and Enzyme Linked Immunosorbent Assays (ELISA). The disease is largely attributed to *Crryptosporidium parvum* based on microscopic identification that relies on oocyst morphology. However, most *Cryptosporidium* species and diverse genotypes are indistinguishable by direct microscopy, IFAT or ELISA (Tzipori & Ward, 2002). Genetic techniques, including the amplification of target genes by Polymerase Chain Reaction (PCR), are used in most investigations to help in determining the possible sources of infection. Molecular analysis has clearly identified that the predominant *Cryptosporidium* species infecting humans are two main species namely, *C. hominis* ((previously known as human genotype) and *C.parvum* that appear to have independent reproductive cycles (Morgan *et al.*, 1999b). The former is transmitted predominantly between humans while the latter cycles between humans, domestic and wild mammals (Fayer *et al.*, 2000). Molecular analysis has also led to the identification of *C.canis*, *C.felis*, *C.meleagridis*, *C.muris*, *C.parvum* 'pig' type and 'cervine' type infecting both immunocompromised and immunocompetent persons, apart from their natural hosts (Pieniazek *et al.*, 1999; Morgan *et al.*, 2000a; Alves *et al.*, 2001b; Xiao *et al.*, 2001; Xiao *et al.*, 2002a; Gatei *et al.*, 2002a; Ong *et al.*, 2002). Other *C.parvum* genotypes such as 'pig' type, 'monkey' type, 'marsupial' type, and 'ferret' type have been identified in their respective animals (Morgan *et al.*, 1999d).

The potential of *Cryptosporidium* as an opportunistic parasite in HIV/AIDS and the recent reports of major outbreaks of cryptosporidiosis in the United States (USA) and the United Kingdom (UK) due to contaminated potable water supplies (Smith & Rose, 1990; Graczyk *et al.*, 1997) indicate that *Cryptosporidium* should be regarded as a major public health problem.

Molecular techniques have gained much more attention lately as they offer precise identification of *Cryptosporidium* species. However, to date, no studies have characterized *Cryptosporidium* isolates from Saudi Arabia, significantly only three reports have been published on the occurrence of *Cryptosporidium* spp identified by microscopy only (Khan *et al.*, 1989; Bollbol, 1992; Al-Braiken *et al.*, 2003). The aims of our present study are; 1) a determinations of the prevalence, seasonality and factors which may increase the risk of *Cryptosporidium* infection in children, 2) assignment to species and genotypic identification of *Cryptosporidium* parasites, and 3) determination of the sero-prevalence of infection and related risk factors in an adult populations in the Jeddah city of Saudi Arabia.

The initial identification was by microscopy with two different staining techniques; ZN and Auramine Phenol (AP). Confirmation was by using ELISA and PCR, while species and genotyping were by Restriction Fragment Length Polymorphism (RFLP) analysis. Two different gene loci were used, the hyper-variable region of the Small Subunit ribosomal Ribonuclic Acid (SSU rRNA or 18S rRNA) and the *Cryptosporidium* Oocyst Wall Protein (COWP). Sub-genotyping identification of *C.parvum* and *C.hominis* isolates was performed by amplifying the glycoprotein sporozoite antigen gp/15/45/60 gene locus. The PCR amplicons for the three gene fragments were then confirmed by gene sequencing. For the antibody detection, Western Blot (WB) analyses using two different antigen namely the 15-17 KDa and 27 KDa proteins was used.

CHAPTER TWO

LITERATURE REVIEW

2.1. HISTORICAL PERSPECTIVE

The first individual to establish the genus *Cryptosporidium* and to recognise its multispecies nature was Ernest Edward Tyzzer, who described the type species, *C.muris* from the gastric glands of asymptomatic laboratory mice (Tyzzer, 1907). He later published a more complete description of the life cycle and the recognition of asexual and sexual modes of reproduction followed by spore (oocyst) formation in which there were four naked sporozoites, and subsequently described a second species, also from laboratory mice (Tyzzer, 1912). The name *C.parvum* was proposed for this parasite and it differed from *C.muris* not only by infecting the small intestine rather than the stomach but also because the oocysts were smaller (Tyzzer, 1912; Upton &Current, 1985). The medical and veterinary significance of this protozoan was described in 1955 as a cause of severe diarrhoea in turkeys and *C.meleagridis* was proposed as a new species (Salvin, 1955). The first human case of *Cryptosporidium* was, however, described in 1976 as a case of severe diarrhoea in a 3-year old immunocompetent girl (Nime *et al.*, 1976). Six new cases of *Cryptosporidium* infection in humans were subsequently reported in the literature between 1976 and 1981, most of them in association with some form of immune deficiency (Meisel *et al.*, 1976; Lasser *et al.*, 1979; Weisburger *et al.*, 1979; Stermmermann *et al.*, 1980). By 1980 it was well accepted that *C.parvum* was a serious primary cause of outbreaks as well as of sporadic cases of diarrhoea in certain mammals (Tzipori, 1988b). Moreover, Tzipori and Others (1980) reported the first case of cryptosporidiosis in a normal immunocompetent adult.

Following the initial discovery of *Cryptosporidium*, this parasite was commonly confused with other apicomplexan genera, especially members of the coccidian genus *Sarcocystis*. Because many *Sarcocystis* spp have oocysts with thin walls that often rupture, releasing free sporocysts, and because each sporocyst contains four sporozoites

like *Cryptosporidium* oocysts, a variety of named and unnamed species were assigned to the genus (Dubey & Pande, 1963; Duszynski, 1969).

It was not until 1982 that cryptosporidiosis was noted by the Centers of Disease Control and Prevention (CDC) as a significant human disease. In 1984, 58 cases had been reported to the CDC. Of which 18 cases were immunocompetent patients and 40 had various immunological abnormalities, the most common of which was AIDS, occurring in 33 patients (Navin & Juranek, 1984).

The disease was then identified in 12 adult male homosexual patients presenting with overwhelming watery diarrhoea and all had HIV/AIDS. Since then, and with the onset of the AIDS epidemic, *Cryptosporidium* has emerged as a life-threatening intractable cholera-like disease in this population (Current *et al.*, 1983; Forgacs *et al.*, 1983; CDC, 1994). Indeed, *Cryptosporidium* has also been recognised as a very common cause of acute diarrhoea in children, especially in developing countries and among clusters of normal immunocompetent adults (Current *et al.*, 1983). In 1993, it reached the public domain when it became widely recognized as the most serious, and difficult to control, cause of waterborne-related diarrhoea following the massive outbreak in Milwaukee (Mackenzie *et al.*, 1995).

2.2. LIFE CYCLE

Unlike other coccidia, *Cryptosporidium* spp have a monoxenous life cycle that can be completed in one host (Levine, 1984). It has six major developmental stages confined to the micovillous region of the host's intestinal epithelial cells. The life cycle begins with the ingestion of oocysts by a suitable host; oocysts excyst to release four naked sporozoites in the gut through a suture line located along one side of the wall. The sporozoites then infect epithelial cells and initiate asexual development (Tzipori, 1988a). The process of excystation is thought to be facilitated by pancreatic enzyme and bile salts. Other workers in experimental models found that at body temperature the main substances stimulating excystation are sodium taurocholate and trypsin (Crawford & Vermund, 1988)

The sporozoites attach to the enterocyte and penetrate into the cell starting the asexual multiplication (merogony) cycle. This invasion process is likely to involve molecules discharged from parasite organelles (rhoptries, micronemes, and denes granules) found at the apical end of the sporozoites (Clark, 1999). These sporozoites are directly attached superficially to the luminal surface of the small intestine epithelium (Fayer *et al.*, 1990). They attach to the host cell by their anterior pole, followed by invagination of the host cell plasma membrane, which extends around the surface of the sporozoite and eventually completely surrounds it, leading to formation of a Parasitophorous Vacuole (PV) bounded by a parasitophorous vacuolar membrane (PVM), in which the parasite undergoes further development in a unique intracellular but extracytoplasmic location. Unlike any other coccidia, however, *Cryptosporidium* has, in addition, a unique structure known as the feeder organelle membrane, which directly separates the cell and parasite cytoplasm. The sporozoites become the haploid trophozoites (type I meront); each haploid nucleus contains 8 chromosomes consisting from 10.1-10.4 kbp of Dioxy-ribonucleic Acid (DNA) each (Fayer *et al.*, 1997). The type I meront undergoes repeated nuclear divisions producing 6 or 8 nuclei, each of which becomes type I merozoite. This type may infect neighboring enterocytes and may, however, be triggered to produce type II meronts which undergo nuclear divisions to form 4 type II merozoites.

Once the type II meront ruptures the released merozoites penetrate the microvillous border of the intestinal epithelial cell and give rise to the sexual developmental stages. The merozoites differentiate into microgametes (male stage), and macrogametes (female stage) that was fertilize to produce zygotes, which, after two asexual divisions, form the cysts wall, containing four naked sporozoites (Navin & Juranek, 1984; Upton & Current, 1985). The wall is believed to be two-layered with an inner thin wall and an outer thick wall. It is suggested that the 80% of the oocysts that develop a thick wall then undergo sporulation while still within intestinal brush border. The other 20% of the oocysts fail to form the outer thick wall, and are covered only by the inner thin wall which can sporulate in the gut, a characteristic quite distinct from other coccidia. The

production and release of these oocysts within the same host are believed to be the key to autoinfection, a phenomenon observed by Tyzzer (1912).

The thick wall oocysts are excreted in the faeces of the host and subjected to the rigours of the environment until they are rendered noninfectious or are ingested by a susceptible host wherein they initiate a new cycle of infection. The infective dose required to initiate infection is small with an ID_{50} of 132 oocysts, and does as low as 30 oocysts have been able to establish infection in a normal immunocompetent host in human volunteers studies (Dupont *et al.*, 1995). This cycle is thought to take between 12-14 hours to complete (Fayer *et al.*, 1997) and the prepatent period varies with the host and species from 2-13 days (Gut *et al.*, 1991).

While oocysts remain viable after exposure to disinfectants such as sodium hypochlorite and sodium hydroxide, their infectivity is destroyed by ammonia, formal saline and freeze-drying conditions. Temperature extremes have been shown to render oocysts noninfectious; heating aqueous suspensions of oocysts to 45 °C, 55°C, and up to 73.7°C rendered oocysts noninfectious for mice (Fayer, 1994; Harp *et al.*, 1996). In contrast, an earlier study reported that oocysts held at 37°C lost infectivity within 5 days, those kept at 15 to 20°C lost infectivity within 2 weeks and those stored at 4°C lost infectivity within 2 months (Sherwood *et al.*, 1982). On the other hand freezing aqueous suspensions of oocysts at-70°C for 1 hour or -20°C for 24 hours or longer also rendered oocysts non-infectious for mice (Sherwood *et al.*, 1982; Fayer & Nerad, 1996).

2.3. TAXONOMY OF *CRYPTOSPORIDIUM* SPP

Cryptosporidium is a genus belonging, taxonomically to the phylum Apicomplexa, class Sporozoasida, subclass Coccidiasina, order Eucoccidoirida, suborder Eimeriorina and family Cryptosporidiidae (Levine, 1985). This 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 coccidians develop in the gastrointestinal tract of vertebrates during part or the whole of their life cycle and there are two groups. One group has an extra-intestinal asexual development and is tissue cyst forming. This group includes the genera *Besnoitia*, *Sarcocystis*, *Neospora,* and *Toxoplasma* (Dubey *et al.*, 1990). The other group develops only in the gastro-intestinal or respiratory mucosa without the formation of tissue cysts and this includes the genera, *Eimeria*, *Cyclospora*, *Isospora,* and *Cryptosporidium.*

One major reason for the long disputes over the taxonomy of *Cryptosporidium* is the difficulty in fulfilling the definition of biological species. The classical definition of species as groups of inter-breeding natural populations reproductively isolated from other groups is difficult to apply to many organisms like *Cryptosporidium*, because it is very difficult to conduct genetic crossing studies with many *Cryptosporidium* spp (Xiao *et al.*, 2004). Oocyst morphometrics alone are not entirely adequate for the description of new species of *Cryptosporidium*, as the oocysts are among the species with the smallest exogenous stages, and differences are more difficult to differentiate, especially by light microscopy (Morgan *et al.*, 1999d). Other characteristics must be included in the taxonomic description.

In many cases experimental transmission followed by light and electron microscopy of endogenous stages has proven useful. In contrast, Current and Colleagues (1986) published an account of the life cycle of *C.baileyi* in chicken and pointed out the morphological differences in oocyst structure between *C.parvum* and *C.baileyi.* Moreover, he showed that *C.baileyi* possessed a third type of merogonous stage not seen in *C.parvum.* However, Xiao and Others (2004) suggested that a strict requirement

for life cycle studies in all taxonomic works seems impractical because distinct species of *Cryptosporidium* might have similar endogenous development.

Host specificity was one of the earliest ways in which *C.andersoni* in cattle was distinguished from the morphologically similar *C.muris* in rodents and this was by the fact that the former species was never infectious for mice (Lindsay *et al.*, 2000). However, the assumed lack of ability of *C.homini* to experimentally infect animals such as calves, lambs and pigs with was apparently premature, since recent studies have clearly shown that calves, lambs and piglets can be infected with this species (Akiyoshi *et al.*, 2002; Ebeid *et al.*, 2003).

Infectivity has sometimes been used to characterize and compare various *Cryptosporidium* isolates. Infectivity can be a good general indicator of host susceptibility and oocysts viability and many infectivity studies have been published (Okhuysen & Chappell, 2002; Pereira *et al.*, 2002).

Biochemical differences can potentially be used as one criterion in defining *Cryptosporidium* spp as RFLP of genomic DNA (O'Donoghue, 1995), isoenzyme analysis (Awad-El-Kariem *et al.*, 1998), protein or carbohydrate surface labelling of oocysts or sporozoites (Tilley & Upton, 1991a; Nina *et al.*, 1992), and two dimensional gel electrophoreisis (Mead *et al.*, 1990) have all been used in an attempt to define both interspecific and intraspecific differences in *Cryptosporidium* spp. For example, differences in protein electrophoresis profiles between bovine *C.parvum* isolates and *C.wrairi* gave strong support to the validity of *C.wrairi* as a separate species (Tilley *et al.*, 1991b).

In recent years molecular characterization of *Cryptosporidium* has helped to clarify the confusion in *Cryptosporidium* taxonomy and to validate the existence of multiple species in each vertebrate class. As a result, several new species of *Cryptosporidium* have been named. Thus, *C.andersoni* from cattle, *C.canis* from dogs, *C.hominis* from humans and *C.molnari* from fish were all established by using multiple parameters that included morphology, developmental biology, host specificity, histopathology, and/or

molecular biology (Fayer *et al.*, 2001; Alvarez-Pellitero & Sitja-Bobadilla, 2002; Morgan-Ryan *et al.*, 2002). Table 2.1. Shows the current valid species of *Cryptosporidium*. Table 2.1. Shows the valid *Cryptosporidium* species and their major (original) and minor (additional) hosts (Xiao *et al.*, 2004).

Table 2.1. Valid *Cryptosporidium* species.

Species	Major host	Minor host
C.muris	Rodents, bactrian camels	Human, rock hyrax, mountain goats
C.andersoni	Cattle, bacterian camels	Sheep
C.parvum	Cattle, sheep, goats, human	Deer, mice, pigs
C.hominis	Humans, monkeys	Dugongs, sheep
C.wrairi	Guinea pigs	-
C.felis	Cats	Humans, cattle
C.canis	Dogs	Humans
C.meleagridis	Turkeys, humans	Parrots
C.baileyi	Chicken, turkeys	Cockatiels, quails, ostriches, ducks
C.galli	Finches, chickens, capercallies , grosbeaks	-
C.serpentis	Snakes, lizards	-
C.saurophilum	Lizards	Snakes
C.molnari	Fish	-

2.4. *CRYPTOSPORIDIUM SPECIES*

There is overlapping in the species infecting different hosts. The following is a review of the species infecting a range of hosts including mamlas, birds, reptiles and fish.

2.4.1. *Cryptosporidium* species of mammals

Mammals represent the largest group of animals known to be infectable by *Cryptosporidium* spp. The taxonomy of *Cryptosporidium* in mammals has been the subject of great dispute since 1980. Moreover, it is now obvious that there is enormous biological and genetic diversity in mammalian *Cryptosporidium*. *C.muris* has been observed frequently in the gastric glands of laboratory mice but not wild mice and its oocysts measure about 7 by 5μm (Tyzzer, 1907). *C.parvum*, the most frequently reported species in mammals, was first found in mice (Tyzzer, 1912). It was differentiated from *C.muris* based on its smaller oocyst size (5.0 by 4.5 μm) and its location only in the villi of the small intestine. *C.andersoni* infects the abomasums of

cattle and produces oocysts morphologically similar to *C.muris*; *C.andersoni* was recognized early to be poorly infective not only to nonbovine hosts but also to cattle (Lindsay *et al.*, 2000). *C.canis* has been observed in the faeces of dogs worldwide, with oocysts measuring 4.9 by 4.7 μm. These oocysts were morphologically indistinguishable from those of *C.parvum* (Fayer *et al.*, 2001). The first report of *C.felis* in cats which included a description of the oocysts from the faeces (5 by 4.5 μm) and its pathogenicity was by Iseki (1979). *C.wrairi* from the guinea pig was identified by Vetterling and Others (1971); only small guinea pigs were usually found to be infected and the infection was not associated with diarrhoea. *Cryptosporidium* parasites infecting humans, previously designated *C.parvum* human genotype or genotype 1, have been delineated to a separate species called *C.hominis*, based on molecular and biological differences (Morgan-Ryan *et al.*, 2002). *C.hominis* is morphologically identical to *C.parvum* (5 by 4.7 μm), but they have different biological activities in cell culture (Hijjawi *et al.*, 2001). However, the number of isolates studied in animal and culture models has been small.

There are probably many other cryptic *Cryptosporidium* species in mammals, all of which were previously assumed to be *C.parvum*. Thus far, nearly 20 *Cryptosporidium* genotypes with uncertain species status have been collectively found in pigs (two genotypes), sheep, horses, cattle, rabbits, marsupials, oppossums (two genotypes), ferrets, foxes, deer (two genotypes), muskrats (two genotypes), squirrels, bears and deer mice (Xiao *et al.*, 2002b). The genetic distances between these *Cryptosporidium* parasites are greater or comparable to these between the known *Cryptosporidium* species and, moreover, cross-transmission studies have shown biological differences among some of these genotypes (Enemark *et al.*, 2003)

2.4.2. *Cryptosporidium* species of Birds

Cryptosporidium infections have been found in over 30 species of birds (Lindsay *et al.*, 1990; Sreter & Varga, 2000b). Significantly, only three avian *Cryptosporidium* spp have been named. *C.meleagridis* infects the small and large intestine with an oocyst measurement of 4.5 by 4 μm (Slavin, 1955). *C.baileyi* is also found in the small and large intestine but can infects the respiratory tract. The oocysts from this species significantly bigger than those from *C.meleagridis*, with measurements of 6.2 by 4.6 μm (Current *et al.*, 1986). The third is *C.galli* which infects only the proventriculus; the oocysts of this species are larger than those of other avian species of *Cryptosporidium* and measure 8.25 by 6.3 μm (Xiao *et al.*, 2004).

2.4.3. *Cryptosporidium* species of Reptiles

Reptiles, especially snakes, are affected most severely by cryptosporidiosis due to the chronic nature of the infection. *C.serpentis* was isolated and named by Levine in1980 and has a distinguishable oocysts (6.2 by 5.3 μm) which differentiates it from the other species infecting reptiles. *C.saurophilum* in lizards, which was named following an extensive study of the faeces from 220 wild and captive lizards of 67 different species (Koudela & Modry, 1998). More recently this parasite was found among 9 of 24 *Cryptosporidium* isolates from monitors, iguanas, and geckoes (Xiao *et al.*, 2004).

2.4.4. *Cryptosporidium* species of Fish

Two named species of *Cryptosporidium* have been found in fish. Most stages of *C.molnari* were found to be at the surface of epithelial cells in the stomach and intestine with oocysts measuring 4.7 by 4.4 μm (Alvarez-Pellitero & Sitja-Bobadilla, 2002). The second species is *C.nasorum* which was named solely on the basis of presumed host specificity and there is no measurement of oocysts available (Xiao *et al.*, 2004).

2.5. PHENOTYPIC AND GENOTYPIC VARIATION IN *CRYPTOSPORIDIUM* SPP

Considerable phenotypic and genotypic variations occur among and within *Cryptosporidium* isolates. Within the group of *C.parvum* there are presently increasing reports of variations in the phenotypic expression of the different isolates. These differences have been recognised based on infectivity for other animals (O'Donoghue, 1995), pathogenicity (Fayer & Ungar, 1986), and antigenicity (McDonald *et al.*, 1991). Italian workers demonstrated the behavioural differences between isolates of *Cryptosporidium* from AIDS patients who were exhibiting mild and severe cryptosporidiosis, and these differences in clinical presentation were thought to reflect the intrinsic characteristics of the parasite strain (Pozio *et al.*, 1992). A study by Okhuysen and Others (1999); three distinct *C.parvum* isolates were identified with different infectivities; differences in the infective dose, attack rate and duration of diarrhoea. A further study confirmed that there were differences in the mean oocyst infective doses among three different *Cryptosporidium* isolates (Dupont *et al.*, 1995).

Analysis of *Cryptosporidium* species using different genetic markers has been used extensively (Bonnin *et al.*, 1996; Carraway *et al.*, 1996). PCR techniques have found broad applicability because their sensitivity permits the analysis of DNA from minute amounts of parasite material (Widmer, 1998a). A range of different genetic loci have been used specifically for the molecular identification or differentiation of *Cryptosporidium* samples. These include the SSU rRNA (18S rRNA), COWP, and the Heat Shock Protein 70 (HSP 70) (Fayer *et al.*, 2000). Among other methods, DNA sequencing and PCR-RFLP have been widely employed for the genotypic identification of *Cryptosporidium* isolates (Gasser *et al.*, 2003). For example isoenzyme analysis, PCR-RFLP, and sequence analysis of a wide range of unlinked loci from different geographic locations have all demonstrated consistent genetic differences between the cattle genotype of *C.parvum* and *C.hominis* (Awad-El-Kariem *et al.*, 1995; Rochelle *et al.*, 1999; Blears *et al.*, 2000; Gobet & Toze, 2001; Sulaiman *et al.*, 2001; Morgan-Ryan *et al.*, 2002).

2.6. *CRYPTOSPORIDIUM* SPECIES INFECT HUMANS

2.6.1. *Cryptosporidium hominis*

The first two cases of human cryptosporidiosis were reported in 1976 (Meisel *et al.*, 1976; Nime *et al.*, 1976). Since then, thousands of human infections have been documented in 95 countries (Casemore *et al.*, 1997; Fayer *et al.*, 2000; McLauchlin *et al.*, 2000). *C.hominis* is widespread in the environment and commonly infects the small intestine and colon of humans (Casemore *et al.*, 1997). Studies in Australia, the USA, Thailand, Switzerland, Kenya and South America have reported *C.hominis* to be the most common *Cryptosporidium* species infecting humans (Morgan *et al.*, 1998b; Sulaiman *et al.*, 1998; Morgan *et al.*, 2000a; Tiangtip & Jongwutiwes, 2002; Gatei *et al.*, 2002b). *C.hominis* is traditionally considered non-infective for mice, rats, cats, dogs, and cattle (Widmer, 1998a; Widmer *et al.*, 2000; Giles *et al.*, 2001). However, there have been reports of non-human infection of *C.hominis*; one report identified *C.hominis* in a dugong (*Dugong dugon*) (Morgan *et al.*, 2000b), another reported *C.hominis* infection in a lamb (Giles *et al.*, 2001) and piglets can also be infected experimentally with at least some *C.hominis* isolates at high doses (Ebeid *et al.*, 2003). Oocysts of *C.hominis* (5.0 by 4.2μm) overlap in size with *C.parvum* bovine genotype oocysts (5.2 by 4.3 μm). However, a molecular epidemiological study of *Cryptosporidium* in children in Lima, Peru, reported that the duration of oocyst detection in stools was significantly longer and the intensity of oocyst shedding was significantly higher for infection with *C.hominis* than for infection with *C.parvum* (Xiao *et al.*, 2001).

2.6.2. *Cryptosporidium parvum*

C.parvum is the most frequently reported species in mammals. As mentioned above this species was originally differentiated from *C.muris* based on its smaller oocyst size and its location only in the villi of the small intestine (Tyzzer, 1912). Recent molecular characterisation, however, has shown that there is extensive host adaptation in *Cryptosporidium* evolution, and many mammals or groups of mammals have host-adapted *Cryptosporidium* genotypes, which differ from each other in both DNA

sequences and infectivity (Xiao *et al.*, 2004). Thus, these genotypes are clearly being delineated as distinct species and this includes *C.hominis* (previously known as human genotype), and *C.canis* (the dog genotype of *C.parvum*). Other genotypes have been associated with the mouse, pig, bear, deer, marsupial, monkey, muskrat, skunk, cattle, and ferret (Xiao *et al.*, 2002 b). *C.parvum* is known to cause infection in humans and various ruminants under natural conditions, and mice, rats, and dogs as experimental infections (O'Donoghue, 1995; Fayer *et al.*, 2000). It is very difficult to differentiate between *C.hominis* and *C.parvum* solely by light microscopy as there is a size overlap of oocysts between the two species. A recent study in the UK, which examined 1,705 faecal samples from humans and reported distinct variations in the distribution of *C.parvum* (which was more common in the spring) and *C.hominis* (more common in late summer and autumn) (McLuchlin *et al.*, 2000). Most European studies have reported that *C.parvum* is more common in patients with cryptosporidiosis than *C.hominis* (McLauchlin *et al.*, 1999; Alves *et al.*, 2001a; Guyot *et al.*, 2001; Pedraza-Diaz *et al.*, 2001). However, a large case control study conducted in the North West Region of England and Wales has recently demonstrated that higher proportion of infections was with *C.hominis* and geographical differences can clearly occur (Hunter *et al.*, 2004).

More recently, *C.parvum* pig genotype was identified in an AIDS patient, although the patient was not severely immunosuppressed as determined on the basis of the Cluster of differentiation of T lymphocyte ($CD4^+$ T) cell count (533cell / μL) (Xiao *et al.*, 2002a). Immunosuppression may not be a prerequisite for *Cryptosporidium* pig genotype infection in humans, as other non- *C.parvum* species such as *C.meleagridis*, *C.canis*, and *C.felis* initially have been found in HIV-positive persons and were subsequently found in immunocompetent persons (Pieniazek *et al.*, 1999; Xiao *et al.*, 2001; Pedraza-Diaz *et al.*, 2001). 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 a patient in British Columbia (Ong *et al.*, 2002).

2.6.3. *Cryptosporidium meleagridis* and *Cryptosporidium baileyi*

C.meleagridis occurs naturally in turkeys and infects the small and large intestine, but can experimentally infect chicken, rabbits, cattle, and immunosuppressed mice (Sreter *et al.*, 2000b). Oocysts are morphologically similar to *C.parvum*, *C.felis* and C.wrairi infecting the small intestines. *C.meleagridis* has been identified in many geographic regions; for instance *C.meleagridis* has been reported in turkeys in the Czech Republic, Romania, Scotland, and the USA (Fayer *et al.*, 2000). This species is now considered to be the third most common *Cryptosporidium* parasites in humans. The first documented human cases of *C.meleagridis* were in two patients with HIV from Kenya and Switzerland (Morgan *et al.*, 2000a). This species was also reported in children in Peru with prevalence as high as the bovine genotype of *C.parvum* (Xiao *et al.*, 2001); again it was identified in HIV-infected patients in Thailand (Gatei *et al.*, 2002b). In a molecular epidemiological analysis of *Cryptosporidium* spp in the UK, in 1,705 faecal samples, *C.meleagridis* was identified in only 0.3% of the immunocompetent people (McLuauchlin *et al.*, 20000).

Human infection with the second avian species *C.baileyi* seems to be rare. One case of *C.baileyi* infection in an HIV infected patient from Australia has been reported (Ditrich *et al.*, 1991); however, the lack of molecular typing makes the diagnosis inconclusive.

2.6.4. *Cryptosporidium felis* and *Cryptosporidium canis*

The validity of *C.felis* was in doubt for some years after its identification, but recent molecular characterisations at the SSU rRNA, Internal Transcribed Spacer 1 (ITS-1) HSP70, COWP and actin loci support the concept of *C.felis* as a valid species (Morgan *et al.*, 1998c; Morgan *et al.*, 1999a). *C.canis* (*C.parvum* 'dog' genotype) has been observed in the faeces of 'dogs' worldwide; however, current genotyping techniques have confirmed that the *C.parvum* 'dog' genotype is a species distinct from the *C.parvum* commonly found infecting humans (Fayer *et al.*, 2001).

The first documented cases of human infection with *C.canis* and *C.felis* were from immunosuppressed patients (Pieniazek *et al.*, 1999). A study on *Cryptosporidium*

parasites from patients in the UK; there were four identified infections with *C.felis*, two of which were in immunocompetent patients (Pedraza-Diaz *et al.*, 2001). In the same study one case of *C.canis* was identified in immunocompetent person. A study from Italy confirmed the presence of *C.felis* in HIV infected patient (Caccio *et al.*, 2002). Subsequent authors have identified *C.felis* and *C.canis* in HIV-infected patients in USA, Switzerland and Thailand (Morgan *et al.*, 2000a; Gatei *et al.*, 2002b).

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 of the parasites in this genus.

2.6.5. *Cryptosporidium muris*

Six different cases of human infection with this species have been identified since its identification in 1907. Three of the cases were confirmed by microscopy and molecular analysis. One case of *C.muris* infection was identified in an HIV-infected child in Thailand and the other were in HIV-infected adults in Kenya and Peru (Tiangtip & Jongwutiwes, 2002; Gatei *et al.*, 2002a; Palmer *et al.*, 2003). The finding of this species in two healthy Indonesian girls was not confirmed by using molecular analysis (Katsumata *et al.*, 2000). In France, *C.muris* showed it to be reported in an immunocompromised patient; however, the sequence analysis of the SSU rRNA was more similar to that of *C.andersoni* (Guyot *et al.*, 2001). Most of these studies have identified *C.muris* in HIV-infected patients, which suggest that immunosuppression may produce a unique susceptibility to infections by unusual *Cryptosporidium* species and genotypes from the environment.

Lately, species other than *C.hominis* and *C.parvum* are increasingly being isolated from humans. This has been partly due to improved diagnosis using techniques able to distinguish the different species and genotypes (Morgan *et al.*, 1999d).

2.7. EPIDEMIOLOGY

Globally, *Cryptosporidium* has now been recognised as a common and highly infectious human enteric pathogen and ranks as one of the four most common aetiological agents of diarrhoea being found most commonly 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 collection criteria, the diagnostic tests used, availability of facilities, and reporting systems (Casemore *et al.*, 1997). However, prospective population-based studies have, in general, been consistent with the overall data available from laboratory surveys (Griffith, 1998). While the point prevalence may be low, there is a high life time incidence rate as theoretically everyone is at risk of acquiring cryptosporidiosis due to its ubiquitous nature, wide range of animal reservoirs and high infectivity (Griffith, 1998).

The reported prevalence rates of *Cryptosporidium* spp among children with and without diarrhoea in developing countries range from 6.1-40.9% and 0-7.5% respectively which are generally higher than those in the developed countries which range from 2.6-22% and 0-2.4% (Guerrant, 1997). Studies have been carried out in US day care centres, UK, Spain, and France, and the reported prevalence rates were respectively 6-65%, 1.4%, 10% and 2% (Rodriguez- Hernandez *et al.*, 1996). Other studies have been carried out in some developing countries with prevalence rates of 10.3 %, 3.5 %, 18 %, and 6.8 % in Pakistan, Bangladesh, Zambia and Nepal (Sherchand & Shrestha, 1996; Bhattacharya *et al.*, 1997; Nchito *et al.*, 1998; Iqbal *et al.*, 1999).

There are few published reports of studies on cryptosporidiosis in the Middle East. Between 6.7% and 19% of the symptomatic children investigated in Jordan (Nimri & Hijazi, 1994) and Gaza (Sallon *et al.*, 1991; Sallon *et al.*, 1994) were found to be infected with *Cryptosporidium* spp. Cross -sectional studies in Kuwait (Daoud *et al.*, 1990; Iqbal *et al.*, 2001), Egypt (Khashba *et al.*, 1989; Mikhail *et al.*, 1989; Stazzone *et al.*, 1996), Sudan (Robinson *et al.*, 1986) and among Bedouin children in Israel (Dagan *et al.*, 1991) revealed prevalence of 1.6%-10%. In Saudi Arabia the reported prevalence rates were 1 %-32 % in symptomatic children from Dammam (Khan *et al.*, 1988), Riyadh (Bolbol, 1992) and Jeddah respectively (Al-Braiken *et al.*, 2003). Table 2.2. Summaries prevalence studies of cryptosporidiosis in developing and various Middle Eastern countries. The prevalence rates of *Cryptosporidium* infection varies from region to region and also from report to report from the same area. These differences have been attributed to differences in the types of patient studied, whether they are hospitalised or out-patients, the immune status of the patients, the age distribution of the population, the season of the year during which the study was carried out, differences in the diagnostic methods used (Crawford & Vermund, 1988; Tzipori, 1988a), geographic variation and socio-economic factors (Casemore *et al.*, 1997).

In developing countries, infection is common in infants aged less than one year during or shortly after weaning, while in developed countries infection is most common in children aged from one to five years, with a secondary peak in young, mainly urban, adults (Casemore, 1990; Palmer & Biffin, 1990). A relative increase in incidence in adults is often seen in waterborne outbreaks (Meinhardt *et al.*, 1996). Adults under 45 years of age are also a risk. It has been theorised that, since this group often cares for young children, they are at risk of secondary transmission from infected children (Griffith, 1998) or they have not yet acquired age related immunity; conversely, there is no apparent geneder-related disposition to infection (Morgan *et al.*, 1999d).

Seroprevalence studies show much higher rates of infection ranging from 25-35% in developed countries and 42-57% in developing countries with prevalence as high as 64% in Latin America (Casemore, 1990; O'Donoghue, 1995). Nearly all the children living in an urban slum in Brazil were found to be seropositive for the infection. Studies in the USA have shown seropositivity rates of 13% in those aged under five, 38% in those aged from 5-13 years and 58% in adolescents (Fayer *et al.*, 1997). In Anhui and rural China 50% of 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).

There is evidence of seasonal peaks of cryptosporidiosis in several studies worldwide particularly in spring and autumn (Clavel *et al.*, 1996; Sorvillo *et al.*, 1998; Inungu *et al.*, 2000). However, published data is contradictory; higher rates have been reported in cool months (Weitz *et al.*, 1988), hot months (Dagan *et al.*, 1991) or the rainy season (Hart, 1999). In a three-year prospective study of diarrhoeal disease among children in Guinea Bissau, the prevalence of cryptosporidiosis was 9.1% in the rainy season compared with 5.1% during the dry season (Molbak *et al.*, 1993).

Other groups at risk of acquiring *Cryptosporidium* infection, include malnourished children (Molbak *et al.*, 1993), a range of immunosuppressed individuals including AIDS patients, transplant recipients, patients receiving chemotherapy for cancer, institutionalised patients and patients with other immunosuppressive infectious diseases (Fayer *et al.*, 2000).

Table 2.2. Prevalence of *Cryptosporidium* infection in children in a number of developing countries.

Country	Study type	Prevalence
Australia (Assadamongko *et al.*, 1992)	Hospital patients	1.4% diarrhoea
Bangladesh (Bhattacharya *et al.*, 1997)	Case-control	3.5% diarrhoea
Brazil, (Newman *et al.*, 1999)	Longitudinal study	25% diarrhoea 4% non-diarrhoea
Egypt, (Risk & Soliman, 2001)	Hospital patients	13.9% malnourished 5.6% control
Ethiopia, (Mersha & Tiruneh, 1992)	Hospital patients	9% diarrhoea
Gambia, (Adegbola *et al.*, 1994)	Longitudinal	9% diarrhoea 3% non diarrhoea
Gaza, (Sallon *et al.*, 1991)	Prospective survey	19%
Guatemala, (Bern *et al.*, 2000)	Hospital patients	1.2% diarrhoea
Guinea Bissau, (Molbak *et al.*, 1990)	Hospital patients	6-17.6% seasonal
India, (Nath *et al.*, 1999)	Cross-sectional	7.2% diarrhoea
Indonisia, (Katsumata *et al.*, 1998)	Hospital & community	2.8& diarrhoea 1.4& non diarrhoea
Jordan, (Nimri & Hijazi, 1994)	Community	4% diarrhoea
Jordan, (Youssef *et al.*, 2000)	Hospital patients	1.5% diarrhoea
Kuwait, (Iqbal *et al.*, 2001)	Hospital patients	10% diarrhoea
Mexico, (Miller *et al.*, 1994)	Hospital patients	9.4% rural 29.6% urban
Nigeria, (Okafor & Okunji, 1996)	School children	39.5% diarrhoea 24.2% non-diarrhoea
Pakistan, (Iqbal *et al.*, 1999)	Hospital patients	10.3% diarrhoea 3.3% non diarrhoea
Saudi Arabia, (Khan *et al.*, 1988)	Hospital patients	1% diarrhoea
Saudi Arabia, (Bolbol, 1992)	Hospital patients	1% diarrhoea
Saudi Arabia, (Al-Braiken *et al.*, 2003)	Cross-sectional	32% diarrhoea 4.7% non diarrhoea
Sudan, (Robinson *et al.*, 1986)	Hospital patients	6.1% diarrhoea
Sudan, (Adam *et al.*, 1994)	Cross-sectional	16% diarrhoea
Turkey, (Akyon *et al.*, 1999)	Hospital patients	3.5% diarrhoea
Uganda, (Tumwine *et al.*, 2003)	Cross-sectional	25% diarrhoea 8.5% non-diarrhoea
Venezuela, (Chacin-Bonilla *et al.*, 1997)	Hospital patients	11.2% diarrhoea

2.8. TRANSMISSION

The oocyst is the stage transmitted from an infected host to a susceptible host by the faecal-oral route. The epidemiology of cryptosporidiosis is complex, involving both direct and indirect routes of transmission from animals to man and from person to person (Casemore *et al.*, 1997). Cryptosporidiosis has been reported worldwide and is common in man, in livestock animals and in wildlife. It also has many biological and epidemiological features that promote transmissibility and complicate control measures. These include: oocysts are excreted fully infective in very large numbers and are generally environmentally robust; the low ID_{50} of 132 (Okhuysen *et al.*, 1999); common disinfectants are not active against *Cryptosporidium* oocysts; the wide host range and geographical distribution; and direct faecal-oral route of transmission. Transmission patterns vary from region to region, perhaps due to a multiplicity of factors and interactions between the parasites, host and environment (Casemore, 1990; Fayer *et al.*, 1997). Generally, transmission is highest at the start of the rainy season when survival of oocysts and their dissemination is easier. In the UK the peaks incidences are in spring, and late autumn to early winter, coinciding with peak rainfall and increased farming activities (Clavel *et al.*, 1996; Hart, 1999).

2.8.1. Zoonotic transmission

Peri-domestic animals, including pets, are reservoirs of infection. Infected animals excrete large numbers of oocysts, sometimes up to 10^{10} daily, for up to two weeks after disappearance of symptoms (Tzipori *et al.*, 1982a). Conversely, since many species of animals are known to harbour the parasite, infection can be transmitted from peri-domestic animals. Zoonotic infection by direct contact with mammalian livestock, especially lambs and calves, is common, particularly in urban children visiting educational farms (Evans & Gardner, 1996). *Cryptosporidium* is suspected to be a major cause of transient diarrhoea among animal handlers (Moon & Woodmansee, 1986; Rahman *et al.*, 1996), including veterinarians (Anderson *et al.*, 1982). Indirect transmission, especially through water, is also common. The widespread epidemic of foot and mouth disease in the UK during 2001, and consequent implementation of

control measures, led to a rapid decline in cryptosporidiosis, estimated at 35% overall, in the human population (Smerdon *et al.*, 2003).

Lately, there have been reports of other zoonotic species such as *C.muris*, *C.meleagridis*, *C.felis*, *C.canis*, 'pig'and 'cervine' genotypes of *C.parvum* infecting humans (Xiao *et al.*, 2001; Pedraza-Diaz *et al.*, 2001; Gatei *et al.*, 2002a; Ong *et al.*, 2002; Xiao *et al.*, 2002a). While it is clear these species are of animal origin, their transmission routes are not yet documented and it is not yet known what proportion of cases of human cryptosporidiosis is of zoonotic origin.

2.8.2. Food borne transmission

The role of *Cryptosporidium* spp as a foodborne pathogen has not been well documented. Epidemiological features of this parasitic protozoan lead to the assumption that the incidence of cryptosporidiosis due to contaminated food is under-estimated. Reports of food related outbreaks are few, difficult to document due to limitations in sensitivity of diagnostic tests and greatly under-reported. Oocysts of *C.parvum* have been detected in oysters, clams and mussels, although, in no case these associated with outbreaks of cryptosporidiosis in humans (Fayer *et al.*, 1999; Graczyk *et al.*, 1999). Epidemiological evidence from the UK has shown that consumption of specific foods such as offal and raw fresh sausage is associated with *Cryptosporidium* infection (Casemor, 1990). Infection is most common in naïve persons with no previous exposure to cryptosporidiosis (Laberge *et al.*, 1996).

Few documented reports however, have been published. In Maine, USA, apples from the ground near a cattle pasture were used to make cider at an agricultural fair; 160 attendees developed cryptosporidiosis (Millard *et al.*, 1994). Oocysts from the attendees had genotypic characteristics implicating a bovine genotype of *C.parvum* (Peng *et al.*, 1997). A cryptosporidiosis outbreak involving 50 school children was associated with milk from a local, small-scale producer in the UK using an on farm-pasteurise (Gelletli *et al.*, 1997).

Mechanical transmission by cockroaches and flies has also been documented which increase the risk from food-borne contamination (Graczyk *et al.*, 1999).

2.8.3. Person to person transmission

Person to person spread of *Cryptosporidium* is one of the most common modes of transmission. Children still wearing diapers who attend child-care centres are at especially high risk, either through intimate play or because of careless diaper-changing practices. Infections acquired by children in child-care or other settings are often transmitted to caregivers as well as to other children and adult family members. Outbreaks tend to occur in day-care centres (CDC, 1984), as household cross-infections (Current, 1994; Fayer *et al.*, 1997), nosocomial infection (Koch *et al.*, 1985), and in custodial institutions (Keusch *et al.*, 1995).

Prospective studies have shown that asymptomatic childhood carriage of *Cryptosporidium* is common, and unsuspected child-to-child transmission may be important in endemic disease (Pettoello-Mantovani *et al.*, 1995). In endemic areas, person–to-person transmission through poor standards of hygiene perpetuates cryptosporidiosis in the community (Casemore, 1990; Rahman *et al.*, 1990). Many outbreaks have been reported in Spain, Portugal and South Africa (Walters *et al.*, 1988; Melo Cistino *et al.*, 1988; Clavel *et al.*, 1996). Moreover, in the USA, *Cryptosporidium* has been implicated in many day-care centre outbreaks. A survey in a day-care centre in Georgia found cryptosporidiosis in 49% of children and 13% of staff members (Tangermann *et al.*, 1991). In Pennsylvania, cryptosporidiosis was detected in 65% of cases from an outbreak, and from 55% of cases in Michigan (CDC, 1984). Thirty-one households in Fortaleza, Brazil, with an index case of cryptosporidiosis in a child under 3 years of age, were studied prospectively by Newman and Colleagues (1994). Secondary cases occurred in 58% of the households and the overall secondary transmission rate was 19%.

Cross infection from patient to patient or to hospital or caring staff members has been reported (Baxby *et al.*, 1983; Casemore *et al.*, 1994; Weber & Rutala, 2001). Hospital

employees and patients are at special risk of acquiring cryptosporidiosis, as AIDS patients in hospital may excrete large numbers of oocysts. Despite the small inoculums needed to cause *Cryptosporidium* infection, there are few case reports in the literature suggesting nosocomial patient-to-patient transmission. Ravn and Others (1991) described a nosocomial outbreak in Denmark, where an incontinent psychotic patient with AIDS and cryptosporidiosis contaminated an ice machine used by patients, visitors, staff, and outpatients. Eighteen of the 60 HIV-infected patients developed cryptosporidiosis and 8 of them died later as a result of this nosocomial exposure. Serological evidence of *Cryptosporidium* in hospital staff after exposure to a patient with chronic cryptosporidiosis suggested that transmission of *Cryptosporidium* is possible from patients to health care workers (Koch *et al.*, 1985). In the UK, there have been reports of leukaemic children contracting cryptosporidiosis in hospital and in isolation ward in North Wales; five nurses contracted the infection from an HIV-infected patient (Foot *et al.*, 1990; Casemore *et al.*, 1994). In Argentina, an outbreak among patients in a renal unit resulted in 11 of 14 patients and one staff member contracting cryptosporidiosis from one infected patient (Roncoroni *et al.*, 1989). Additionally, Navarrete and Others (1991) described a paediatric hospital outbreak in which 82% of children exposed to an infant with cryptosporidiosis and AIDS developed cryptosporidiosis.

2.8.4. Sexual transmission

Several large studies have suggested the role of sexual transmission, but were unable to confirm cryptosporidiosis by this route. In a prospective cohort study of people with AIDS, homosexual men were more likely to develop cryptosporidiosis (4.1%), than were Intravenous Drug Users (IVDUs) (1.3%) (Pedersen *et al.*, 1996). In Spain 26% of homosexual men had enteropathogens including *Cryptosporidium* compared to 12% of the IVDUs (Moreno *et al.*, 1994). These studies very strongly suggest that cryptosporidiosis can be acquired sexually, but does not conclusively prove it since transmission could, theoretically, be related to other associated behaviour.

2.8.5. Air-borne transmission

Respiratory symptoms are more common in cryptosporidiosis than in other diarrhoeal diseases. Although there have been no proven cases of airborne transmission in humans the concept was suggested by investigators in 1987 (Hojlyng *et al.*, 1987), because the infective dose is low, and the respiratory tract is easily infected. There are, however, numerous reports of high rates of cough or other pulmonary symptoms in children and immune compromised persons with cryptosporidiosis (Griffiths, 1998).

2.8.6. Traveller's diarrhoea

Most cases of traveller's diarrhoea are caused by bacteria, and are acute in nature and resolve within 5-10 days after the onset of symptoms. The most common intestinal protozoa-infecting traveller's are *Giardia lamblia*, *Cryptosporidium* spp, and *Entamoeba histolytica/dispar*, although a smaller proportion of infections are due to *Microsporidia* and to *Isospora*. In recent years, *Cyclospora cayetanensis* has been recognised as a causative organism of chronic diarrhoea in returning travellers (Okhuysen, 2001). Cryptosporidiosis has been identified as a cause of diarrhoea in travellers returning from areas of poor sanitation (Soave & Ma, 1985). A survey of US Peace Corps volunteers who were residing in West Africa demonstrated a 13.6% increase in sero-prevalence during a 2-year period of stay (Ungar *et al.*, 1989).

2.8.7. Water-borne transmission and outbreaks

Water presents a major route of transmission, both in drinking water and through recreational use (Meinhardt *et al.*, 1996; Rose *et al.*, 2002). The waterborne route of infection has generated serious concerns among public health and regulatory organisations with regards to the safety of drinking water in both the developed and developing world (Moore *et al.*, 1994). Important sources of *Cryptosporidium* oocysts are discharges of untreated and treated domestic sewage and agricultural run-off (Medema & Schijven, 2001). *Cryptosporidium* has been isolated worldwide from many water sources such as rivers, streams, and swimming pools. In nearly all the reported waterborne outbreaks in developed countries, the quality of the water after treatment by

the water utility met community or state standards for acceptable drinking water (LeChevallier *et al.*, 1996). These outbreaks indicate that water treatment standards did not adequately protect against waterborne cryptosporidiosis (CDC, 1995).

In drinking water treatment plants using conventional filtration, a summary of studies indicated that oocysts were found in finished water between 3.8 and 33.3% of the time at concentrations from 0.1 to 48 oocysts per 100 L (Rose *et al.*, 2002). In the USA in 1988, surface water was used by over 155 million people in 6000 community water systems, of which 23% provided unfiltered water to 21 million people. Protection from infectious agents relied solely on disinfection. What is not well documented about *Cryptosporidium* is the viability, species, and source of the oocysts found in tap water (Fayer *et al.*, 2000). Although, waterborne infections in individuals are difficult to document, outbreaks of cryptosporidiosis linked to drinking water clearly confirm that viable *Cryptosporidium* oocysts can enter and pass through the drinking water purification processes.

The first reported waterborne outbreak of cryptosporidiosis was reported in 1984 and was attributed to faecal contamination of a public artesian well in Texas, USA (D'Antonio *et al.*, 1985). In 1987 an outbreak, first recognised as a dramatic increase in gastroenteritis among college students, affected about 13,000 of 64,900 residents in Carroll County, Georgia, USA (Hayes *et al.*, 1989). In the spring of 1993, the largest recorded outbreak of waterborne disease of any kind occurred in Milwaukee (MacKenzie *et al.*, 1995). More than 403 000 of about 1, 600, 000 people in the greater Milwaukee area developed cryptosporidiosis after consuming contaminated drinking water. The source of contamination remained uncertain; but recent genotypic analysis of four of the Milwaukee isolates indicated consistency with *C.hominis* (Peng *et al.*, 1997). Similar outbreaks in the UK and Northern Ireland have been linked to inadequate treated drinking water (Patel *et al.*, 1998; Furtado *et al.*, 1998; Hunter *et al.*, 2001; Glaberman *et al.*, 2002). Although reports of waterborne outbreaks in developing countries are few, this is more likely to be due to under-reporting (Fayer *et al.*, 1997). Waterborne outbreaks have been demonstrated in Lusaka, Zambia where over 500 people presented with diarrhoea lasting over 2 weeks (Kelly *et al.*, 1997). Many other

waterborne outbreaks have been documented with patterns similar to those mentioned above. Table 2.3. Demonstrates some selected waterborne outbreaks.

Table 2.3. Selected waterborne outbreaks from different countries.

Country	Number of cases	Suspected cause
Ayrshire, UK (Smith *et al.*, 1989)	27	Spring water
Carroll County, Georgia (Hayes et al., 1989)	13, 000	Treated water
Loch Lomond, UK (Barer & Wright, 1990)	442	Loch water
Milwaukee, WI (MacKenzie *et al.*, 1995)	403, 000	Lake water
North West, UK (Furtado *et al.*, 1998)	63	Drinking water
Northern Ireland (Glaberman *et al.*, 2002)	230	Drinking water
Northern Italy (Pozio *et al.*, 1997)	294	Water tanks
Texas (D'Antonio et al., 1985)	5900	Well water
Warrington, UK (Bridgman *et al.*, 1995)	47	Tap water
Waterloo, Canada (Rose *et al.*, 1997)	1000	Tap water

Few studies have characterised the species isolated from waterborne outbreaks (Kramer *et al.*, 1996). So far, *C.hominis*, *C.parvum*, 'cervine' genotype and *C.meleagridis* have been identified as contaminating water for human consumption (Xiao *et al.*, 2000a; Glaberman *et al.*, 2002; Ong *et al.*, 2003). Of the 14 outbreaks investigated in North America, 71% were caused by *C.hominis* and 29% by *C.parvum* (Xiao *et al.*, 2000a). Similarly, most of the UK outbreaks were caused by the *C.hominis* (Homan *et al.*, 1999; McLauchlin *et al.*, 1999).

Swimming pools are a significant risk for recreational transmission (Meinhardt *et al.*, 1996; Furtado *et al.*, 1998; Rose *et al.*, 2002). In the past years, reported outbreaks of cryptosporidiosis related to recreational water have affected over 10,000 people (Fayer *et al.*, 2000). Frequent faecal contamination, coupled with the resistance of oocysts to chlorine, and its low infectious dose, have all facilitated transmission (Carpenter *et al.*, 1999). Routine use of recreational waters by incontinent persons, including diapered children and toddlers, increases the potential for waterborne transmission (Korich *et al.*, 1990). It may be prudent for immunocompromised individuals to avoid freshwater

streams or pools where other humans have bathed, as direct faecal contamination may occur (Griffiths, 1998).

Recently, major resources have been allocated to ensure that all water for human consumption is free of *Cryptosporidium* and other protozoa such as *Giardia*. However, implementation of practical, affordable, and effective methods for the elimination of the parasite from drinking water remains an elusive goal (Leav *et al.*, 2003). In the UK, control strategies are aimed at removing or killing *Cryptosporidium* oocysts and *Giardia* cysts. Current guidelines require water supply organisations to ensure that there is less than 1 oocyst per 10L of water (Bustamante *et al.*, 2001; Craik *et al.*, 2001). In other countries, including some states in the USA and Canada, treatment and monitoring for cryptosporidiosis is less stringent (Payment *et al.*, 2000).

The current strategy for elimination of *Cryptosporidium* species from the public water supply involves preventing contamination of water sources, physical removal of the organisms, and chemical or physical disinfection. However, these methods are only capable of reducing the number of oocysts, not eliminating the parasite from the water supply (Leav *et al.*, 2003). Water treatment methods such as sweep flocculation engulfment; ultraviolet and other radiation have been developed to rid water of *Cryptosporidium* (Bustamante *et al.*, 2001; Craik *et al.*, 2001). Decontamination of water through parasite inactivation using ozone and monochloramine is applied especially to swimming pools where contamination is with faecal material (Hirata *et al.*, 2001). However, these sophisticated approaches, have not been widely applied for various reasons, including financial and health-related concerns (Rose *et al.*, 2002; Leav *et al.*, 2003).

Stringent measures including PCR, flow-cytometry, colorimetric methods, ELISA, electrorotation and immunofluorensce have been undertaken to analyse water quality (Bustamante *et al.*, 2001). However, these processes are expensive and may not always ensure safe water. While stringent measure 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. Monitoring the

water supply may not predict an outbreak or rule it out as a source of infection, as the distribution of oocysts is not homogenous and varies from source, treatment and distribution points (Casemore *et al.*, 1997). Failure to detect oocysts in water supplies should clearly not be taken as a guarantee of water safety (Griffiths, 1998).

It is very important to consider the effect of global climate change, such as the frequency of heavy rainfall prior to many outbreaks. Reports suggest that this may lead to an increased incidence in food borne and waterborne infections, and increased monitoring and control may need to be considered after a period of heavy rain (Rose *et al.*, 2002).

2.9. EPIDEMIOLOGY IN PEOPLE WITH HIV-INFECTION

Cryptosporidium infection in HIV/AIDS patients brought into focus the potential threat of this parasite among this group of patients in the early 1980s. Generally, as the immune status decreases as shown by a decrease in the $CD4^+$ T cell counts, the proportion of patients infected increases (Chappell & Okhuysen, 2002). The prevalence of cryptosporidiosis is higher in immunocompromised hosts, particularly in patients with AIDS. Cryptosporidiosis is generally one of the most common causes of chronic diarrhoea in AIDS patients.

The prevalence of infection ranges from 4% in developed countries up to 50% in hospitalized patients in developing countries (Mwachari *et al.*, 1998). In a study in Mali, cryptosporidiosis was associated with chronic diarrhoea and dehydration resulting in over 40% mortality (Pichard *et al.*, 1990). A study among South African children admitted in a hospital showed a prevalence of 11.9% of cryptosporidiosis infection (Van den Ende, 1986). The development of cryptosporidiosis in HIV-infected patients usally carries a poor prognosis. The survival time after diagnosis was found averaged 15 weeks without antiretroviral therapy (McGowan *et al.*, 1993). Patients with higher $CD4^+$ T cell counts did significantly better (Moss *et al.*, 1995). Similar findings have been reported from Senegal, Tanzania, and Ethiopia (Mengesha, 1994; Dieng *et al.*, 1994; Gomez *et al.*, 1995).

Immunocompromised individuals can be infected with more genetically diverse parasite populations (Widmer *et al.*, 1998c). In addition to *C.hominis* and *C.parvum*, *C.canis*, *C.felis*, *C.meleagridis*, *C.muris*, and the pig genotype of *C.parvum* have been found in AIDS patients with associated clinical symptoms (Morgan *et al.*, 2000a; Pedraza-Diaz *et al.*, 2001; Gatei *et al.*, 2002a; Xiao *et al.*, 2002a).

2.10. RISK FACTORS

Malnutrition appears to be a risk factor for cryptosporidiosis and this is perhaps due to the effect of nutrition on immunity leading to an increase in susceptibility to infection. In malnutrition, cellular immune function is the part of the immune system most affected. Thus, cryptosporidiosis is found in this group with increased frequency, as in other conditions affecting cellular immunity such as AIDS (Sallon *et al.*, 1988). The association between chronic cryptosporidiosis, persistent diarrhoea and malnutrition is not well established, although several reports indicate that children with malnutrition are more likely to develop persistent diarrhoea (Tzipori & Ward, 2002). A study of children in Mexico showed a 40% higher risk of the cryptosporidiosis among poor urban children (Miller *et al.*, 1994). In the University Hospital in the West Indians, 23.7% of stools from malnourished children with diarrhoea contained *Cryptosporidium*. As mentioned earlier, that there is evidence of seasonality in transmission. Several studies indicate seasonality, particularly, in the spring and autumn, which do not necessarily both occur in any one locality (Casemore, 1990). Seasonal peaks may also reflect agricultural practices and exposures such as lambing and calving and application of faeces as fertiliser. Temporal patterns may also be attributed to such factors as seasonal attendance in child-care centres and ensuing transmission associated with these centres (Clavel *et al.*, 1996).

Infection and subsequent disease development is also linked to parasite dynamic factors that may contribute to infectivity including selectivity for human hosts, differences in susceptibility to environmental stress, drug susceptibility, and variability in virulence (Okhuysen *et al.*, 1999). It has long been known that animals and humans with

cryptosporidiosis have variable expressions of disease severity and immune responses. While some of these variations in the expression of disease may be due to differences in host susceptibility to infection, these variations may also be due to intrinsic diversity in isolate pathogenicity (Okhuysen & Chappell, 2002). Experimental studies have shown that different strains have different viability, virulence and minimum infective doses (Okhuysen *et al.*, 1999). The well-adapted strains may require very low infective doses of oocysts but may present a less virulent disease in terms of clinical severity and secondary transmission (Chappell *et al.*, 1996).

2.11. IMMUNE RESPONSE

In normal hosts, cryptosporidiosis is self-limited, while in immunocompromised hosts, such as patients with AIDS, persistent diarrhoea frequently develops. Thus, the immune response appears to play a critical role in limiting infection. Cell-mediated immunity appears to be the major component of the immune response to *Cryptosporidium* infection. Although there is prominent humoral response to infection, the exact role of antibodies in host defence against *Cryptosporidium* infection is unclear (Leav *et al.*, 2003). Initial studies focused on the role of antibody. Polyclonal and monoclonal antibodies were noted to neutralise the sporozoite stage and to prevent infection when given orally (Riggs *et al.*, 1994). Similarly, bovine immune colostrum can limit infection in *vitro*, in murine models, and in some human cases (Nord *et al.*, 1990). Recent studies have focused on responses to the 15-17 KDa and 27KDa antigens (Moss *et al.*, 1998; Okhuysen *et al.*, 1998). Antibodies to the 15-17KDa and 27KDa antigens can be considered as very good markers of infection, since the antigens are recognised by IgA, IgG, and IgM serum antibodies in many different species (Reperant *et al.*, 1994). There is also data that suggests that antibody has little or no role in controlling infection (Taghi-Kilani *et al.*, 1990).

The correlation between the decreased number of $CD4^+$ T cells and the risk of *Cryptosporidium* infection is evidence of the critical role that such cells play in immunity (Riggs, 2002). Studies that use murine models in which $CD4^+$ T cells were deficient confirmed the central role of these cells in adaptive immunity to

cryptosporidiosis (Riggs, 2002). Furthermore, chronic cryptosporidiosis in AIDS patients correlates with a decrease in T cell function. Patients with $CD4^+$ T counts of > 180 cells mm^3 usually have a self- limiting infection, whereas most patients with counts < 140 cells mm^3 develop severe and persistent infection (Current & Bick, 1989). From lymphocyte/cytokine depletion or adoptive transfer studies in SCID mice and other models, the central importance of $CD4^+$ T cells, Interferon- γ (IFN-γ) and Interlukin-12 (IL-12) in resistance to and recovery from *Cryptosporidium* infection has become clear (Riggs, 2002). The cytokines IFN-γ, and IL-12, have also been shown to be protective against *Cryptosporidium* infection in laboratory models (Lean *et al.*, 2002).

Innate immune mechanisms may also be important in resistance to *Cryptosporidium* infection. $CD4^+$ T cells appear to be the source of IFN- γ in the adaptive immune response, and these cells have been shown to limit experimental infection (Riggs, 2002). In contrast, Cluster of Differentiation 8 T lymphocyte ($CD8^+$ T) cells, natural killer cells, and the Tumour Necrosis Factor-α (TNF-α) do not appear to play a significant role in host defence against *Crryptosporidium* infection (Chen *et al.*, 1993; Rohlman *et al.*, 1993).

2.12. CLINICAL MANIFESTATIONS

2.12.1. Intestinal disease

Essentially, all humans are susceptible to *Cryptosporidium* infection, although people with serological evidence of previous infection seem to be more resistant (Chappell *et al.*, 1999). The clinical presentation of cryptosporidiosis depends mainly on the host response; however, the clinical features of cryptosporidiosis are non-specific and one cannot make a diagnosis based solely on the symptoms (Griffith, 1998). *Cryptosporidium* has a pre-patent period of 2-13 days with a mean of 9 days and a median of 6.5 days as has been demonstrated in *vitro* in animal models and in healthy volunteers (Current & Reese, 1986; Gut *et al.*, 1991; Chappell *et al.*, 1996).

In the immunocompetent patients the commonly presented symptoms include acute self-limiting diarrhoea, vomiting, abdominal cramps, fever, general malaise, flatulence

and weight loss. In about 20% of outbreak cases, patients experience a recurrence that may be mild within one week or for up to three months (MacKenzie *et al.*, 1995; Chappell *et al.*, 1999). The duration of illness in immunocompetent hosts is variable, lasting from several days to five weeks (Jokipii & Jokipii, 1986).

In children, the diarrhoea usually lasts 2 weeks (Wolfson *et al.*, 1985; Current & Garcia, 1991; Molbak *et al.*, 1993). Oocysts are excreted in stools for 1-2 weeks after resolution of the symptoms (Jokipii & Jokipii, 1986). However, some exposed patients are asymptomatic. In a study in Naples, they identified asymptomatic cryptosporidiosis in 12.5 % of subjects. In experimentally infected humans, one third of the subjects who ingested oocysts had no diarrhoea (DuPont *et al.*, 1995). Asymptomatic carriage may also be higher in areas of low socioeconomic development. A study in Peru found a higher asymptomatic carriage rate than symptomatic infection among children (Checkley *et al.*, 1997).

Several published reports have shown that cryptosporidiosis may have devastating long-term effect in children, such as shortfalls in linear growth and weight gain (Checkley *et al.*, 1997; Checkley *et al.*, 1998; Agnew *et al.*, 1998). Infected children had long periods of diarrhoeal illness, in some occurring intermittently for up to 21 months and with accompanying fitness deficits. In Brazil, cryptosporidiosis and persistent diarrhoea among children has been correlated with subsequent impairments in physical fitness and diminished cognitive function (Guerrant *et al.*, 1999). In Guinea Bissau, cryptosporidiosis in infants was associated with an increase in all-cause mortality that was independent of differences in soci-economic status and nutritional status (Molbak *et al.*, 1993). It appears that these children with persistent diarrhoea are derived in a cycles of malnutrition, placing them at risk for further diarrhoeal illness, which in turn leads to progressively worsening nutritional status (Lima *et al.*, 2000).

Although the disease is self-limited in most patients, in several other situations cryptosporidiosis can cause significant morbidity and death. In immunocompromised patients, clinical signs range from transient dairrhoea to profuse cholera-like enteric disease that is characterised by severe diarrhoea, abdominal cramps, and severe weight

loss (Kamel *et al.*, 1994; Robinson, 1995; Manabe *et al.*, 1998). with resulting high mortality (Ungar *et al.*, 1990; Blanshard *et al.*, 1992). The risk of acquiring cryptosporidiosis is correlated with lower numbers of $CD4^+$ T cells (Hunter & Nichols, 2002). Transient disease or asymptomatic carriage is common in people with $CD4^+$ T counts of greater than 250 cells /mm^3 (Blanshard *et al.*, 1992). Patients with $CD4^+$ T cells count of less than 50 cells/mm^3 present with profuse cholera like illness and have a great risk of disseminated cryptosporidiosis to other organs such as liver and the Central Nervous System (CNS) (Blumberg *et al.*, 1984; Brady *et al.*, 1984; Vakil *et al.*, 1996). In a cohort of HIV-infected patients studied in Baltimore during 1985-1995, four patterns were described; transient infection, intermittent relapsing infection, cholera-like infection, and chronic infection (Manabe *et al.*, 1998). Profuse diarrhoea leads to severe electrolyte imbalance and shock (Blanshard *et al.*, 1992). A stool volume of up to 7L /day with a loss of up to 17 Liters of body fluid has been reported (Ungar *et al.*, 1990).

In these patients the main histopathological features of cryptosporidiosis include changes to the intestinal mucosa, such as disruption of the epithelial cell barrier, while more extensive infiltration of the lamina propia with inflammatory cells has been noticed (Lumadue *et al.*, 1998). The diarrhoea is typically non-inflammatory and is often profuse, the parasite does elicit a local inflammatory response, and increased production of prostaglandins and several cytokines, in particular IFN-γ production has been well described (Chen *et al.*, 2002). However, the mechanism by which *Cryptosporidium* infection causes diarrhoea remains elusive, especially since no toxin has been detected and the actual disruption of the intestinal mucosa dose not match the severe clinical picture (Moore *et al.*, 1994; Griffiths *et al.*, 1998; Chen *et al.*, 2002).

2.12.2. Respiratory disease

Respiratory infection is common but usually clinically not apparent. In children cryptosporidiosis-related cough is frequent, being reported in one-fifth to one-third of immunocompetent children. Kone and Others (1992) reported that of 250 children in Ivory Coast with cryptosporidial diarrhoea, 77% had profuse diarrhoea, 58% had fever, and 19% had pulmonary symptoms. It has been postulated that transient respiratory

cryptosporidiosis is common in immunocompetent children (Egger *et al.*, 1990). However, this is not surprising given the tens of billions of oocysts that may be excreted during infection, and the ability of the parasite to invade the respiratory tract epithelium (Griffiths, 1998). Pulmonary symptoms have been reported in approximately three-times more often in children admitted to hospital with cryptosporidial diarrhoea than in children with other intestinal pathogens in prospective case-control studies (Egger *et al.*, 1990).

Patients with respiratory cryptosporidiosis usually present with croupy cough, wheezing and dyspnoea. In such cases, oocysts are detectable in sputum, broncho-alveolar lavage and tracheal aspirates (Kocoshis *et al.*, 1984). In humans with AIDS respiratory cryptosporidiosis can rarely be the presenting feature (Mifsud *et al.*, 1994). Respiratory infection in this sub-population is marked by severe persistent cough, copious tracheal secretions and dyspnoea (Kocoshis *et al.*, 1984). In a study from Spain, where a total of 7 out of 34 people with AIDS and cryptosporidiosis had respiratory infection (Lopez-Velez *et al.*, 1995), but only 4 of the 7 had abnormal chest radiographs. In a report from Copenhagen, a total of 8 out of 86 diagnostic bronchoscopes in AIDS patients were positive for *Cryptosporidium* (Jensen *et al.*, 1990). If bronchoscopy samples were routinely examined for *Cryptosporidium*, more cases would be reported in the literature (Griffiths, 1998).

2.12.3. Hepatobiliary disease

Another well described manifestation of *Cryptosporidium* infection in patients with AIDS is cholangitis, which is seen more often with more severe immunosuppression, defined as a $CD4^+$ T cell count of <50 cells/mm^3 (Chen *et al.*, 2002). Gastric cryptosporidiosis and pancreatitis have also been described in patients with AIDS (Hunter & Nichols, 2002). Humans with persistent cryptosporidiosis may develop hepatobiliary or pancreatic duct infection. *Cryptosporidium* was first reported as a cause of cholecystitis in 1983 (Pitlik *et al.*, 1983) and is now identified in up to 60% of patients with AIDS cholangitis (Benhamou *et al.*, 1993; Ducreux *et al.*, 1995). It has been postulated that *Cryptosporidium* infects the biliary tree by spread from the small

bowel via the common bile duct (Kahn *et al.*, 1987). Biliary carriage is common in HIV-infected patients that are less immunocompromised and may be a significant reservoir for the relapse of cryptosporidiosis seen in chronic disease (Casemore *et al.*, 1997).

After the Milwaukee outbreak in 1993, of the 82 HIV-seropositive individuals who developed cryptosporidiosis, only 4 of the 24 (71 %) with biliary symptoms were a live 1 year after the outbreak, compared with the 30 of 58(52 %) without biliary symptoms. However, those with biliary symptoms were more likely to have $CD4^+$ T cell counts < 50 /mm (Griffiths, 1998). Thus biliary disease was associated with more advanced immunocompromised states.

2.13. DIAGNOSIS

Cryptosporidiosis was initially diagnosed via invasive techniques. Intestinal analysis via biopsy, with demonstration of intracellular forms of the parasite, is specific but the diagnosis may be missed because most common sites of infection are less accessible endoscopically (Leav *et al.*, 2003). Numerous methods that have been designed for detecting cryptosporidiosis either through detection of oocysts by staining, immunological assays or immunofluorescence, and molecular techniques (Arrowood, 1997). Oocyst shedding is intermittent in all stages of the infection; therefore, several stool samples may need to be examined before indicating the negativity of the sample. The stool sample can be examined fresh or preserved, in 10% formalin or potassium dichromate (2.5% w/v) (Fayer *et al.*, 1997); a recent report from Thailand has shown that 75% ethanol can preserve the sample even at room temperature (Jongwutiwes *et al.*, 2002).

2.13.1. Staining techniques

Cryptosporidium oocysts were first described in a histological section of gastric mucosa using Romanovsky's stain (Tyzzer, 1907). Subsequently, the first human cases were diagnosed from intestinal biopsy section examined by light or electron microscopy (Meisel, 1976; Nime *et al.*, 1976). However, these procedures are invasive and also

since only a section of the gut is examined it can yield false-negative results. Giemsa was later found to stain oocysts in stool; however, the method has low specificity (Tzipori *et al.*, 1980) as yeasts, which also stain purple like the oocysts of *Cryptosporidium,* can cause diagnostic confusion. In 1981, Henrickson and Pohlenz (1981) introduced the ZN staining technique, which stains the oocysts pinkish-red (4-5 μm in diameter). This has remained the most commonly used procedure for identifying *Crryptosporidium* oocysts in the stool and is 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 such as Modified Cold Kinyoun (MCK) which shortens the staining time to between 30-60 seconds by the addition of 1% tergitol to the carbol fuchsin (Ma, 1988) and the use of cold or hot carbol fuchsin. The safranin methylene blue, which stains the *Cryptosporidium* oocysts orange pink whilst yeasts and other faecal debris stain blue, is said to be more sensitive than the ZN stain (Baxby *et al.*, 1984); however, this technique is not widely used. Fluorescent stains including AP, auramine-rhodamine and auramine-carbol-fuchsin which are among others and are used routinely (Moodley *et al.*, 1991; Tortora *et al.*, 1992). These fluorescence techniques are said to be more sensitive but less specific compared with the ZN stain. Crawford and Vermund (1988) suggested that the samples positive with auramine need to be confirmed with the ZN stain. It is, therefore, useful in screening large numbers of stool samples with low infection rate.

A *Cryptosporidium* identification tests should be specifically requested by the clinician, because it will not be performed as part of a routine examination for ova and parasites. Interpretation of the stained smear requires experience because other organisms in the stool may have similar appearance.

2.13.2. Immunoassay methods

Several immunofluorescent assays and ELISA kits have become commercially available and are used increasingly commonly used for the detection of *Cryptosporidium.* These tests use antibodies against *Cryptosporidium* antigens to detect the parasite in stool specimens. They have the advantage of being more sensitive and less user-dependent

compared to acid fast staining (Fayer *et al.*, 1997). The IFAT can also be used to detect *Cryptosporidium* oocysts in stool specimens. A commercially available kit MeriFluor™ (Meridian Diagnostic Inc, Cincinnati; Ohio) demonstrates sensitivity of 98-99% (Garcia *et al.*, 2000). In comparative studies, the IFAT is both more sensitive and more specific than acid-fast staining techniques (Arrowood & Sterling, 1989; Kehl *et al.*, 1995). However, widespread application of this test has been limited by the additional cost. ELISA-based tests to detect *Cryptosporidium* antigen in stools have also been widely used with sensitivities and specificities comparable to IFAT (Kehl *et al.*, 1995). Agglutination assays using latex beads coated with anti-*Cryptosporidium* antibodies from rabbits immunized with oocyst were found to have low specificity, with excessive false positives (Pohjola *et al.*, 1986).

2.13.3. Molecular techniques

Molecular methods for the detection of *Cryptosporidium* have received much more attention in the last decade and have answered many questions (Fayer *et al.*, 2000). A variety of PCR tests offer alternatives to conventional diagnosis of *Cryptosporidium* for both clinical and environmental specimens (Smith, 1998; Morgan & Thompson, 1998; Wu *et al.*, 2000). Detection of the organism in environmental samples or in sub-clinical cases using conventional techniques such as microscopy is extremely hard as the oocysts may be present in very small numbers.

Different isolates of *Cryptosporidium* possess different antigens, virulence, infectivity, and drug sensitivity. These characteristics were determined by phenotypic tools, which include protein analysis, antigenic diversity and isoenzyme analysis (Current & Reese, 1986; Mead *et al.*, 1990; McDonald *et al.*, 1991; Nichols *et al.*, 1991; Griffin *et al.*, 1992; McLauchlin *et al.*, 1998); but large numbers of oocysts are required to do these tests. Conversely, the application of PCR, in which a very small quantities of genetic material can be amplified to produce large amounts of replicated DNA, has provided researchers with sufficient material further to investigate the variations within the genus *Cryptosporidium* (Morgan & Thompson, 1998). An important advantage of the molecular techniques is that they allow not only for accurate and sensitive detection of

Cryptosporidium but also provide information on the genetic variability of isolates of *Cryptosporidium* (Fayer *et al.*, 2000).

2.13.3.1. Genotypic analysis of *Cryptosporidium* spp

The chromosomes of *C.parvum* have been examined in previous studies. Mead and Others (1988); demonstrated chromosomal size polymorphisms among clinical isolates of *C.parvum* and between *C.parvum* and the avian-derived species *C.baileyi*, using field inversion gel electrophoresis. Of the five *C.parvum* isolates that were analysed, all yielded the same profile consisting of five bands migrating in the 1.4-3.3 Mbp size range. The electrophoretic profile of *C.baileyi* was distinct, and consisted of six chromosomal bands ranging in size between 1.4 and over 3.3 Mbp. Kim and Colleagues (1992); employed orthogonal-field-alternation gel electrophoresis and visualised five bands of size from 0.9 to 1.4 Mbp in *C.parvum*, some of which appeared to contain multiple chromosomes of similar sizes. Five bands were also distinguished by Countour-Clamped Homogeneous Electrophoresis Field (CHEF) in previous studies (Khramtsov *et al.*, 1996; Petersen *et al.*, 1992; Steele *et al.*, 1995). However, Hays and co-authors (1995); who used the CHEF technique, reported eight chromosomes on the basis of comparisons of gels run for different amounts of time; they found the sizes ranged from 0.94 to 2.2 Mbp. Indeed, the *C.parvum* karyotype had remained a rather controversial issue until 1997, when Blunt and Others (1997) described a new characterisation of the *C.parvum* chromosome, combining CHEF electrophoresis with a densitometric scanning of the ethidium bromide-stained chromosomal bands. They confirmed the presence of five chromosomal bands of 1.54, 1.44, 1.24, 1.08, and 1.04 Mbp. Densitometric analysis showed that the largest (1.54 Mbp) and the middle (1.24 Mbp) bands contain multiple chromosomes; the largest band appears to be doublet and the middle band appears to be triplet, and the genome of the *C.parvum,* therefore, consisted of eight chromosomes with a total size of approximately 10.4 Mbp ranging from 1.04 to 1.5 Mbp. The *C.parvum* genome is small for a coccidian (Tilley & Upton, 1997) with ~ 60-70% AT content of the genome (Jenkins & Petersen, 1997; Piper *et al.*, 1998) which seems to be much lower than that of most *Plasmodium* spp (Weber, 1988). Moreover, the low copy and genome-wide distribution of both 5S (four to five copies)

and the 18S rRNA (five copies) (Taghi-Kilani *et al.*, 1994; Le Blancq *et al.*, 1997) is similar to that of several other apicomplexans, such as *Plasmodium*. However, this differs markedly from the usual eukaryotic pattern of hundreds or thousands of tandem copies (Piper *et al.*, 1998).

C.parvum appears to lack the plastid commonly found in apicomplexan parasites (Fayer *et al.*, 1997; Kohler *et al.*, 1997). However, recently a high number of two extrachromosomal virus-like double-stranded (ds)-RNAs were detected in oocysts of several *C.parvum* and *C.hominis* isolates (Khramtsov *et al.*, 2000).

The inability to obtain purified samples of the various developmental stages of the parasite for biochemical studies and the relatively small size and simple organisation of the genome has led to the lack of knowledge of the basic cellular and molecular biology of this pathogen. However, since the genomic DNA sequence encodes all of the heritable information responsible for parasite development, disease pathogenesis, virulence, and immune resistance, a comprehensive knowledge of *Cryptosporidium* genome should provide necessary information for disease prevention and treatment (Liu *et al.*, 1999).

While direct comparison of nucleotide sequences is the ultimate method or 'gold standard' for detecting DNA sequence variation, the identification of consistent markers provides less complex tools for application to large numbers of samples required for epidemiological investigation. Genetic markers for *Cryptosporidium* species have been identified (Awad-El-Kariem *et al.*, 1994; Spano *et al.*, 1997b; Morgan *et al.*, 1999a; Xiao *et al.*, 1999c). Ortega and Others (1991); were the first to examine genetic polymorphisms and confirmed phenotypic characteristics suggesting humans harboured two different isolates, one from calves (*C.parvum*) and other transmitted between humans only (*C.hominis*). Genetic differences between *C.parvum* and *C.hominis* were clearly identified by isoenzyme analysis (Ogunkolade *et al.*, 1993; Awad-Al-Kariem *et al.*, 1995; Awad-El-Kariem *et al.*, 1998). Differences between these species were confirmed by Random Amplified Polymorphic-DNA (RAPD-DNA) analyses (Morgan *et al.*, 1995; Carraway *et al.*, 1996; Deng & Cliver, 1998; Shianna *et al.*, 1998). A study

that analysed 211 faecal specimens positive for *Cryptosporidium* by microscopy used PCR-RFLP analysis of 18S rRNA, COWP, and Thrombo-spondin related adhesion protein-*Cryptosporidium* 1 (TRAP-C1) gene fragments and found 38% were *C.hominis* and 62%, *C.parvum* (McLauchlin *et al.*, 1999). PCR-RFLP analysis of the polytheronine (poly T), COWP gene, Ribonuclease reductase (RNR) and PCR analysis of the 18S rRNA gene was also conducted on *Cryptosporidium* isolates from AIDS patients. Five of the patients excreted *C.hominis* and two excreted *C.parvum* (Widmer *et al.*, 1998c). In each study and at each locus, two electrophoresis profiles were observed and indicated the presence of the two species. A subsequent study reported that sequence and PCR-RFLP analysis of the β-tubulin intron revealed polymorphisms within *C.hominis* and evidence of recombination between *C.parvum* and *C.hominis* (Widmer *et al.*, 1998b). However, others have analysed the same region and found no support for these results (Sulaiman *et al.*, 1999). Despite substantial genetic differences between *C.hominis* and *C.parvum*, little variation is found within these species. Within *C.hominis,* minor differences have been found in the 18S rRNA (Xiao *et al.*, 1998), Thrombo-spondin Related Adhesion Protein-*Cryptosporidium* 2 (TRAP-C2) (Peng *et al.*, 1997), and poly (T) genes (Widmer *et al.*, 1998a). However, this was not the case for the gp15/45/60 gene, which has significant differences between *C.hominis* and *C.parvum* isolates and within different allelic groups (Strong *et al.*, 2000; Leav *et al.*, 2002).

Many tools which have been applied to identify species/genotypes within *Cryptosporidium* and these include the investigation of RAPD-DNA, locus specific amplification by PCR-RFLP and real-time PCR (Amar *et al.*, 2004). The most widely targeted gene loci have been the 18S rRNA (Xiao *et al.*, 1999c) and the COWP gene (Spano *et al.*, 1997b) with the former being the most common target in both prokaryotes and eukaryotes. Other target gene loci also include the ITS1 and Internal Transcribed Spacer 2 (ITS2) (Morgan *et al.*, 1995), the acetyl-CoA synthetase gene (Black *et al.*, 1996), the dihydrofolate reductase-thymidylate synthase (*dhfr-ts*) gene (Gibbons *et al.*, 1998), the 70KDa HSP70gene (Sulaiman *et al.*, 2000), the TRAP-C1 and TRAP-C2 genes (Peng *et al.*, 1997; Spano *et al.*, 1998; Elwin *et al.*, 2001), poly-T (GP 900)

(Spano *et al.*, 1998), (RNR) (Widmer *et al.*, 1998c), Initiation Translation Factor elF-4A (Spano & Crisanti, 2000b) and an unidentified genomic fragment (Bonnin *et al.*, 1996).

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.*, 1999c). However, PCR on the COWP, TRAPC-1 and TRAPC-2 gene loci failed to amplify samples that contained diverse species of *C.parvum* that were later shown to be *C.felis* and *C.canis* with the use of 18S rRNA gene and HSP70 gene fragments analysis (Pedraza-Diaz *et al.*, 2001). While it is evident that some primer amplified all species within the genus (18S rRNA and HSP70), others are more specific such as TRAP-C2, which is specific for *C.parvum* and *C.hominis* (Elwin *et al.*, 2001). Primer pairs must, therefore, be chosen according to the identification being asked; as must the restriction enzymes, which need to be applied, and additional enzymes may be required to differentiate all species.

The main advantage of molecular analysis is in its application to specimens that are unsuitable for investigation by conventional methodologies, and because of its sensitivity and simplicity, PCR-RFLP typing has been extensively applied to the characterisation of *Cryptosporidium* isolates in clinical and epidemiological studies. However, the limited resolution offered by this method has emphasised the need for techniques capable of discriminating among different isolates, even if they belong to the same species. In order to overcome this problem, Microsatellites (non-coding small nucleotide repeats that are present in all eukaryotic organisms) which constitute a rich source of polymorphisms have been used extensively for high-resolution genotyping and mapping (Aiello *et al.*, 1999; Feng *et al.*, 2000). Caccio and Others (2001); identified a polymorphic *Cryptosporidium* microsatellite located within what appears to be a protein coding sequence. More recently, the PCR-coupled mutation scanning method, Single-Strand Confirmation Polymorphism (SSCP), which relies on a sequence-dependent analysis of amplicons, was evaluated for the direct display of sequence variation within and among ribosomal or heat shock gene amplicons (Gasser *et al.*, 2001). Although this method, couple the advantages of species and genotype

identification of *Cryptosporidium*, it has not yet been employed as a molecular-epidemiological tool to screen large numbers of samples for genetic variability.

2.13.3.2. Phylogeny of *Cryptosporidium*

Molecular data based on DNA have become an important in evolutionary studies. Evolutionary changes in morphological and physiological characteristics is so complex, that this approach does not produce a clear-cut picture of evolutionary history and is largely impended by the effects of selection pressure that can lead to identical phenotypes of unrelated organisms developing under similar environments (Kunz, 2002). It is well known that most of the eukaryotic DNA sequence is non-coding and it is not significantly controlled by natural selection. However, analysis can be applied directly to assess not only closely related organisms but also the links between ancient evolutionary occurrences (Wen-Hsiung Li, 1997).

Molecular phylogenetic analyses of the Apicomplexa that have included *Cryptosporidium* have consistently grouped *Cryptosporidium* species as a clade separate from the coccidian taxa with which they are presently classified (Cai *et al.*, 1992; Barta *et al.*, 1997; Carreno *et al.*, 1999). In recent phylogenetic analyses using the 18S rRNA of gregarine parasites of insects, it was found that the genus does not form a monophyletic clade with other intestinal coccidians including *Eimeria*, *Isospora*, *Sarcocystis* and *Toxoplasma*. However, the genus does appear to be a sister group to the gregarine parasites (Barta *et al.*, 1997; Carreno *et al.*, 1999). The gregarine/*Cryptosporidium* clade was separate from another major apicomplexan clades containing the coccidian, adeleids, piroplasms, and haemosporinids indicating that *Cryptosporidium* has a closer phylogenetic association with the gregarine than the coccidians. A study compared 52 complete 18S rRNA sequences representing diverse genera with the phylum Apicomplexa and other related taxa. In this study the species included were from Ciliophora, Dinozoa, Haematozoa, and coccidians against different species of *Cryptosporidium* while species from other genera such as *Caryospora*, *Hepatozoon*, and *Perkinsus* were omitted. The analysis, which used Maximum Likelihood (ML), Neighbour-joining (NJ) and Maximum Parsimony (MP) phylogeny

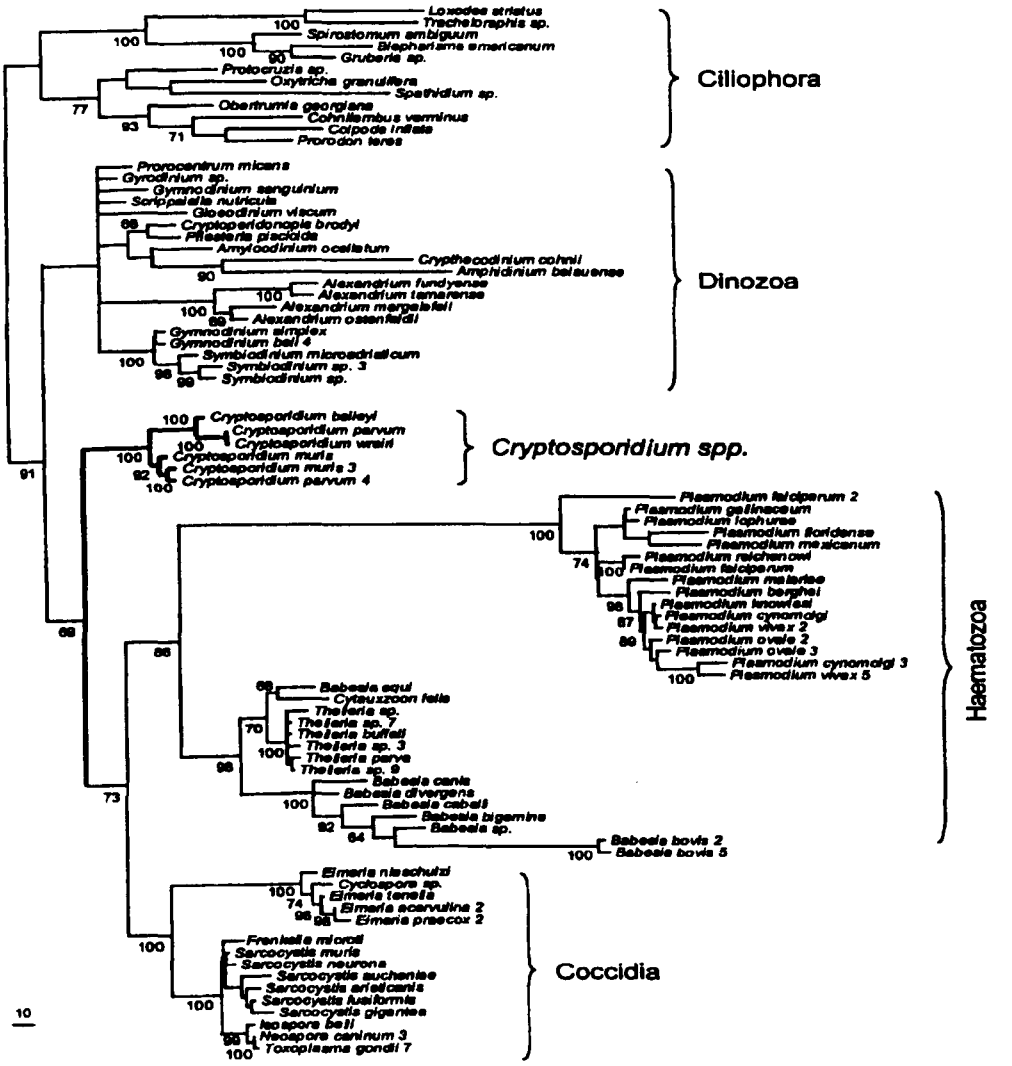
supported the hypothesis that the *Cryptosporidium* genus is a sister-group of the coccidian Haematozoa (bootstrap value of 73%) rather than a member of coccidian class (Zhu *et al.*, 2000). It appears that there may be genuine differences in the genome and the biology of the genus from its closest relatives that set it apart (Coombs, 1999). Although these studies suggest that *Cryptosporidium* may be an early emerging lineage among the Apicomplexa, the bootstrap support appears to be low, suggesting the need for further research (Zhu *et al.*, 2000). Moreover, biochemical, drug testing, ultra-structural data and molecular phylogenetic data are consistent with the emergence of *Cryptosporidium* at the base of the Apicomplexa (Coombs, 1999; Riordan *et al.*, 1999; Zhu *et al.*, 2000). Although the life cycle of *Cryptosporidium* is typically coccidian, i.e.one or more stages of merogeny, a sexual stage producing oocysts and sporulation of oocysts to form sporozoites, it differs from typical euococcidia both in its intracellular location (just beneath the plasma membrane of the enterocyte) and by sporulation (within the gut rather than in the environment) (Fayer *et al.*, 1997). All euococcidia and haematozoa studied to date possess both a plastid organelle and genome (Wilson & Williamson, 1997; Kohler *et al.*, 1997) and elongated mitochondria with cristae in which genes are encoded (Wilson & Williamson, 1997). *C.parvum* appears to lack both a plastid and its genome (Zhu *et al.*, 2000), but has an unusual, ribosome-studded mitochondrion, which lies posterior to the nucleus (Riordan *et al.*, 1999).

Results of molecular and phylogentic analyses from different *Cryptosporidium* isolates confirm the multi-species nature of this genus. Sequence analysis of the 18S rRNA gene has shown nucleotide differences among different *Cryptosporidium* species (Xiao *et al.*, 1999a; Morgan *et al.*, 1999a). Phylogenetic trees based on these sequences indicate that *Cryptosporidium* can be divided into two groups; the gastric parasites (*C.muris, C.andersoni, C.serpentis*) and the intestinal parasites (*C.parvum, C.hominis, C.felis, C.canis, C.wrairi, C.meleagridis*, and *C.baileyi*). The genetic distances between some *Cryptosporidium* spp are comparable to those between other well-established apicomplexan genera and species. Similar conclusions can be reached based on sequence and phylogenetic analyses of two single-copy genes; the HSP70 and the COWP genes (Xiao *et al.*, 2000c).

Extensive genetic diversities are also present among *C.parvum* isolates from cattle, pigs, mice, ferrets, and marsupials. However, the genetic distances among these *C.parvum* genotypes are generally smaller than the interspecies differences among *C.muris, C.baileyi, C.serpentis*, and *C.parvum.*

Figure 2.1. Shows the Maximum Parsimony phylogram inferred from an SSU rRNAdataset containing 52 complete sequences within the phylum Apicomplexa using Dinozoa and Ciliophora as outgroups to root the tree. Note the emergence of *Cryptosporidium* as a sister group of the Haematozoa rather than a number of the coccidea (Zhu *et al.*, 2000).

Figure 2.1.Phylogram of Apicomplexan parasites based on the SSU rRNA gene.



2.14. TREATMENT

Despite decades of research on hundreds of chemotherapeutic and immunotherapeutic agents either in *vitro* or in *vivo* in animals and in clinical trials, there is still no specific therapeutic or preventative modality approved for cryptosporidiosis. Non-specific supportive treatment, including rehydration and nutritional supplementation, remains the mainstay of management of the clinical manifestations of cryptosporidiosis (Tazipori & Ward, 2002). Exhaustive reviews of the numerous chemo-and immunotherapeutic agents evauated for anticryptosporidial activity have been published previously (Blagburn & Soave, 1997; Crabb, 1998). However, the drugs currently indicated in the treatment for cryptosporidiosis give unpredictable outcomes. Paromomycin continues to be one of the few antimicrobial agents that remain consistently in clinical use (Hewitt *et al.*, 2000). A recent report on a patient treated with paromomycin showed effective parasite clearance and resolution of the diarrhoea, despite being heavily immunocompromised (Caccio *et al.*, 2002). A recent approach to therapy has been to use combination chemotherapy. A small open-label study of a combination of paromomycin and azithromycin for four weeks followed by paromomycin alone for eight weeks in 11 patients with AIDS and CD4 counts <100 reported a significant and consistent reduction in symptoms and oocyst excretion (Smith *et al.*, 1998). One of the newer chemotherapeutic agents to be evaluated is nitazoxanide, which has a broad-spectrum anti-parasitological activity against protozoa, nematodes, trematodes and cestodes (Rossignol & Maisonneuve, 1984). In an uncontrolled trials of nitazoxanide in 12 patients with AIDS- associated cryptosporidiosis in Mali, seven individuals had a >95% reduction in oocyst excretion, and in four of these seven patients, eradication or decrease in oocysts excretion was associated with complete resolution of diarrhoea (Doumbo *et al.*, 1997).

The reasons for this remarkable and tenacious resistance of *Cryptosporidium* to various antimicrobial agents are not known. However, other studies propose that *Cryptosporidium* might have some distinct genotypic characteristics that are reflected in the different phenotypic profiles, some of which may confer resistance to most anti-coccidial drugs (Zhu *et al.*, 2000). It is clear that a competent host immune system is

the major factor in resolving cryptosporidiosis. The urgent need for an effective treatment in developing countries against the life-threatening impact of cryptosporidiosis in people with HIV/AIDS has subsided with the use of Highly Active Antiretroviral Therapy (HAART) coupled with antimotility agents (Schmidt *et al.*, 2001), but remains a major issue in poor settings where HAART is not available.

It is presumed that antimicrobial agents must first enter the host cell cytoplasm in order to effectively inactivate intracellular microorganisms. Other mechanisms involve the killing of the host cell containing the intracellular organism (Tzipori & Ward, 2002). The unique location of *Cryptosporidium* below the cell membrane or the PVM and the short life span of the extracellular forms (sporozoites, two generations of merozoites and microgametes); however, make the organism elusive to a wide range of chemotherapeutic and immunotherapeutic agents (Tzipori & Griffiths, 1998).

2.15. NON BACTERIAL DIARRHOEAL DISEASE IN THE MIDDLE EASTEARN

This section describes the protozoal infections causing diarrhoea in the Middle East countries.

2.15.1. Protozoa infection

2.15.1.1. *Entamoeba histolytica*

Infection with the intestinal protozoan *Entamoeba histolytica* is worldwide and approximately 500 million people each year get amoebiasis, but only about 10% present with diarrhoea (Walsh, 1986a). *E.histolytica* has a cosmopolitan distribution although the incidence and morbidity are higher in the tropics (Beaver *et al.*, 1984). It is indistinguishable morphologically from *E.dispar*. However, it can be differentiated by zymodeme patterns, monoclonal antibodies, and DNA probes (Huston & Petri, 1999). Infection with the former results in symptomatic intestinal illness (80% -98%) or invasive disease (2%-20%) and the production of serum antibodies; however, *E.dispar* is considered to be non-pathogenic.

Major routes of transmission are consumption of contaminated water and food or by direct faecal-oral contact (Okhuysen, 2001). The diagnosis may be problematic and mortality may ensue, especially in vulnerable groups such as children, pregnant women, and those receiving treatment with steroids (Walsh, 1986b; Hira *et al.*, 2001). However, without biochemical or immunological differentiation, it can only be presumed that the current records of *E.histolytica* may include a large proportion of cases of *E.dispar* (Beaver *et al.*, 1984). A study in Philippines using PCR differentiate the two organisms showed a 7.3% prevalence of *E.dispar* compared to 0.96% prevalence for *E.histolytica* (Rivera *et al.*, 1998) and similar study using ELISA has shown the predominant of *E.dispar* in Africa and South America (Lawson *et al.*, 2004).

Developing countries have been the major casualties of amoebic infection. Recently, groups at high risk for amoebiasis in non-endemic and developed countries are recent immigrants, institutionalised patients and male homosexuals (Yoshikawa *et al.*, 1999). In the Middle East, surveys have recorded *E.histolytica/dispar* prevalences of 27% in Turkey (Ustun *et al.*, 2003), 22.9% in Jordan (Ali-Shatyeh *et al.*, 1989), 42.3% in Yemen (Kopecky *et al.*, 1992), and 21% in Lebanon (Araj *et al.*, 1996). In Saudi Arabia prevalence of 1.2%-14% have been reported from different cities in the country (Omar *et al.*, 1991a; Al-Madani & Mahfouz, 1995; Al-Braiken *et al.*, 2003) without the differentiation between the two species.

2.15.1.2. *Giardia lamblia*

Giardia lamblia, also known as *G.intestinalis* or *G.duodenalis*, can produce infection in man and may also be a zoonotic agent. This protozoan pathogen is responsible for approximately 100 million infections annually (Okhuysen, 2001). Transmission is through ingestion of the cysts either directly or indirectly through food or contaminated water (Garcia *et al.*, 2000) and this can result in asymptomatic infection, acute self-limited diarrhoeal illness, or chronic gastrointestinal symptoms (Okhuysen, 2001). The infective dose of *G.lamblia* is as few as 10 cysts and this low infectious dose raises the potential risk of waterborne outbreaks associated with *G.lamblia* infection (Bella *et al.*, 1998; Robertson & Gjerde, 2000). It is found worldwide, in temperate and tropical

locations. *G.lamblia* infections occur in children and infants in both developing and developed countries (Pickering *et al.*, 1984; Rauch *et al.*, 1990). In the Middle East several studies have been undertaken to determine the prevalence of *G.lamblia* infection (Ahmed & el Hady, 1989; al-Tukhi *et al.*, 1991; Nimri, 2003; Mukhtar, 1995; Mahmud *et al.*, 1995) showing it to be most common protozoan identified in children.

G.lamblia is also suspected to be a zoonosis. The parasite has been identified among wildlife and in most domestic animals; subsequently wildlife are suspected to be potential reservoir sources for the infection (Dykes *et al.*, 1980). The parasite is also a common cause of protracted diarrhoea in non-immune infecting people visiting endemic areas (Gray & Rouse, 1992).

2.15.1.3. *Isospora belli*

I.belli is endemic in tropical and subtropical environments; It is associated with outbreaks of diarrhoeal disease and it has been implicated as a cause of traveller's diarrhoea (Okhuysen, 2001). Man is the only host and transmission is associated with contaminated water, although that route is not proven. In immunocompetent hosts the clinical features are abdominal pain and diarrhoea; and, it causes prolonged diarrhoea and malnutrition in immunocompromised patients. Isosporiasis is a significant cause of prolonged diarrhoea in such patients, accounting for 10-15% of all cases in area such as India, Zambia and Senegal. A case of isosporiasis in an HIV-infected patient has been described in Dakar, Senegal (Dieng *et al.*, 1994). *Isospora* is known to be a rare cause of childhood diarrhoea. In India, for example, Mirdha *et al.*, (2002), only detected seven cases of symptomatic isosporiosis among 4,112 children with diarrhoea. Few studies have been conducted in the Middle East for detecting this parasite and it was found in prevalence of 3.8% in Egypt (Rezk *et al.*, 2001) and 0.5% in Saudi Arabia (Al-Braiken *et al.*, 2003).

2.15.1.4. *Cyclospora cayetanensis*

Cyclospora cayetanensis is the parasitic agent responsible for human cyclosporiasis. This coccidian parasite was originally identified as a cause of diarrhoea in developing countries such as Nepal and Peru (Okhuysen, 2001). Cases in developed countries have been acquired mostly during outbreaks of infection that have been related to imported raspberries (Herwaldt & Ackers, 1997). The vehicles of transmission are most likely contaminated water and food (Sturbaum *et al.*, 1998).

Cyclosporiasis is an emerging worldwide cause of diarrhoea in immunocompetent people as well as in immunocompromised patients, such as those with AIDS. The symptoms associated with infection with this organism have been reported to resemble those of cryptosporidial infection (Hart *et al.*, 1990; Albert *et al.*, 1994; Shields & Olson, 2003). In general, infection is self-limited in otherwise healthy adults, but diarrhoea can be prolonged and, if untreated, it appears to be more severe than that experienced with *Cryptosporidium* spp (Okhuysen, 2001). In AIDS patients *Cyclospora,* if not treated, may lead to chronic diarrhoea (Ortega *et al.*, 1993; Pape *et al.*, 1994). In the Middle East few reports have been published on the prevalence of this parasite (Rezk *et al.*, 2001; Nimri, 2003; Al-Braiken *et al.*, 2003; Turk *et al.*, 2004).

2.14.1.5. Microsporidia

Microsporidia are small, obligate, intracellular organisms that infects vertebrate and invertebrate hosts. Microsporidia have been sporadically reported as a cause of chronic diarrhoea that occurs after travel in both otherwise healthy adults and HIV-infected patients (Lopez-Velez *et al.*, 1999; Raynaud *et al.*, 1998). The infection in immunocompetent patients is self-limited; however, the infection should be considered in cases of chronic diarrhoea in AIDS patients. The prevalence of infection in selected groups of HIV-infected patients in different countries is in the range of 1.7%-30% (Okhuysen, 2001). In a study in Egypt, Rezk and Others (2001); reported a microsporidial prevalence of 2.4% in immunocompromised patients while this organism was not detected in the immunocompetent group.

2.15.2. Viral infection

Acute gastroenteritis continues to be a major cause of childhood morbidity throughout the world and one of the leading causes of mortality among children in developing countries (Saderi *et al.*, 2002). Rotavirus is the commonest cause of acute watery diarrhoea in children throughout the world (Hart & Cunliffe, 1999; Hart *et al.*, 2002). Rotavirus is acquired via the faecal-oral route. It is responsible for up to 50% of cases of diarrhoeal disease in infants requiring hospitalisation (Hart & Cunliffe, 1997). Moreover, it has been firmly established that rotaviruses are the most important aetiologic agents of dehydrating and gastroenteritis in infants and young children worldwide (Cunliffe *et al.*, 2001).

In developing countries rotavirus infection appears to occur at an earlier age than in developed countries (Cunliffe *et al.*, 1998). In temperate countries there is a distinct seasonal pattern to rotavirus diarrhoea with the majority of the cases being in winter (Hart & Cunliffe, 1997). Many reports have been published in the Middle East reporting high prevalence rates of rotavirus, adenovirus, astrovirus, and coronavirus infection (Amini *et al.*, 1990; Dutta *et al.*, 1990; Akhter *et al.*, 1994; Aithala *et al.*, 1996; Meqdam *et al.*, 1997; Naficy *et al.*, 2000; Saderi *et al.*, 2002; Kurugol *et al.*, 2003).

CHAPTER THREE

PREVALENCE OF *CRYPTOSPORIDIUM* SPP INFECTION IN CHILDREN AND ASSOCIATED RISK FACTORS

3.1. INTRODUCTION

Intestinal parasites are a leading cause of chronic infection in humans worldwide with estimates showing that at least one quarter of the world's population is infected (WHO, 1999). Intestinal parasites present a major public health problem in the developing world. In these countries over 80 % of all deaths are due to infectious and parasitic disease accounting for more than 3 million deaths annually (Al-Madani *et al.*, 1989; WHO, 1999). Protozoa and helminthic intestinal infections are estimated to affect 3.5 billion people worldwide, the majority being in children (Bundy, 1997). Amoebiasis is the second most common cause of death due to a protozoan parasite worldwide. Of the 48 million people estimated to suffer from severe amoebiosis 100,000 die annually (WHO, 1987).

The prevalence of these parasites is usually associated with an unsanitary environment and with the unhygienic habits of the people, and most intestinal parasites have been associated with ill health in such countries. Despite the overall improvements in sanitation the total number of people infected with parasites worldwide is thought to be increasing (Bundy, 1997; Curtis *et al.*, 2000; Scolari *et al.*, 2000), and unlike other parasitic diseases such as malaria and schistosomiasis that cause more specific morbidity, the effect of intestinal parasites infestation in specific communities is less clear (WHO, 1987).

In Bangladesh over 80% of the population have one or more parasites (Khan *et al.*, 1986). In some parts of India rates of *A.lumbricoides* infection have been reported to vary from 80% to 95% (Elkins *et al.*, 1986). However, *G.lamblia* and *E.histolytica/dispar* have been reported less frequently (in about 11%) and *Cryptospridium* spp has a prevalence of 18.9% in the Delhi area, India (Kaur *et al.*, 2002). In Pakistan the prevalence of helminths was found to be 81% with a high rate (48%) of cases of *A.lumbricoides* infection (Ahmed *et al.*, 2003) and rates of

50% and 48.9% for *G.lamblia* and *E.histolytica/dispar* respectively (Siddiqui *et al.*, 2002).

In Yemen, a neighboring country of Saudi Arabia, 53% of the stool specimens submitted to a public health hospital were positive for intestinal parasites (Farag, 1985). Several reports have been published on parasitic infection in Saudi Arabia. A total of 1167 stool specimens collected from 0-6 year old patients attending the King Abdulaziz University hospital in Riyadh, were examined for intestinal parasites. 20.8% were positive with a high prevalenc of *G.lamblia* infection (Bolbol *et al.*, 1989). In 1990, about 30% of the population of 3 closed communities of children in Asir, South Western, Saudi Arabia were found to have symptomatic infections with either *G.lamblia* and/or *E.histylotica/dispar* (Omar *et al.*, 1991b). Stool specimens from 1282 children between 5-13 years attending 10 primary schools for boys in Abha (South West region of Saudi Arabia) were examined for the presence of intestinal parasites, 24.4 % were found to be infected with one or more species with a prevalence of 10% for *G.lamblia* infection (Omar *et al.*, 1991a). Between March and May of the year 2000 stool specimens were collected from 250 children < 5 years from 6 pre-schools, crèches and clinics in the Jeddah city of Saudi Arabia. 47.4 % had at least one species of protozoan parasite with high prevalence rates of *Cryptosporidium* spp (32%) and *G. lamblia* (29%) (Al –Braiken *et al.*, 2003).

Cryptosporidium is now considered to be an important cause of acute childhood diarrhoea in both developed and developing countries but more so in the latter (O'Daonoghue, 1995; Clark, 1999). It is also an important cause of persistent diarrhoea in children especially in developing countries. Previous studies in Saudi Arabia suggest it is present in 1-32% of symptomatic children (Khan *et al.*, 1988; Bolbol, 1992; Al-Braiken *et al.*, 2003). Currently most routine laboratories diagnose *Cryptosporidium* infection by identifying the oocysts in stool samples by using the ZN staining technique, which is said to have a sensitivity and specificity of 86% and 98% respectively (Morgan *et al.*, 1998a). More sensitive methods for diagnosis have been developed, for instance AP staining method, which is also said to be very sensitive; other techniques include the detection of *Cryptosporidium*

copro-antigen in a stool using ELISA, fluorescent antigen detection and PCR techniques (Arrowood, 1997).

This chapter describes the comparison of three different diagnostic methods for identifying *Cryptosporidium* spp in stool; the ZN, AP staining techniques, and ELISA. In addition, it aimed to define the prevalence and seasonality of *Cryptosporidium* infection in children and to define the factors, which may predispose to risk of infection in this population in the Jeddah city, Saudi Arabia. This study also examined all other parasitic infections within the study population.

3.1.1. Study area

Occupying four-fifths of the Arabian Peninsula, the kingdom of Saudi Arabia is the largest country in the Middle East.This study was undertaken in Jeddah City which is located in the western region of the Kingdom and occupies an area of 560km^2. It has one of the main ports, the Red Sea port, which is located midway along the western coast of the kingdom (Figure 3.1). Its location on the ancient trade routes and its status as the seaport and airport for Hajjis visiting the Holy city of Makkah (about 70 km east of Jeddah) have ensured that Jeddah is the most cosmopolitan of all Saudi Arabia's cities. In addition to its marine life, the water of the Red Sea is also a vital commodity, as the city of Jeddah is totally dependent on it for household and industrial supplies. These supply drinking water, which has been purified to a high standard as well as non-potable domestic water. By 1999 the estimated population in the Jeddah area had exceeded 2 million (85% urban and 15% rural). Moreover, the extraordinary growth of Jeddah, demanded by the Kingdom's development programs has been achieved in a remarkably short period. As a result, Jeddah now contains networks of roads, gardens, spacious houses, public and private schools and hospitals and trade centres.

Figure 3.1. Map of Saudi Arabia. The arrow shows the study area location.

3.1.2. Geography and climates

The country has a varied topography; besides the well-known deserts, there is a green mountainous area in its south-western corner. The climate of the kingdom varies from region to region according to its location. Saudi Arabia has no rivers or permanent streams and it is one of the driest countries in the world, with rainfall averaging less than 5 inches per year. Jeddah on the shores of the Red Sea has a hot and humid climate all year-round. Summer is very hot with temperatures reaching 38°C, winter is cooler with an average temperature of 20°C. Between October and April the climate is generally pleasant with cool nights and sunny days. From May to September, on the other hand, temperatures are considerably higher and life without air-conditioning would be very unpleasant.

3.1.3. Health services

All the cities of the Kingdom are well served by government health centres and hospitals as well as that provided by the private health sector. The private sector health facilities have provided health services in co-ordination with, and complementary to, the government health care network throughout the kingdom. The Ministry of Health was able, through its ambitious program over the period from the first development plan (1975) until 1997, to maintain steady progress in the health services field, which is indicated by the increase in the rates of annual growth of the health units and the health manpower.

3.1.4. Health and pilgrimage

The kingdom of Saudi Arabia with the two Holy Mosques, which are the destination for performers of Hajj and Omrah from all over the world, bears the burden of facilitating and taking care of pilgrims. The kingdom gave a high priority to the health of pilgrims and took care of it, from the time of their arrival into the Holy Land until the departure of the last pilgrim. This care was manifested in a substantial increase in the number of physicians working during Hajj season and their distribution across the Holy places. There are at least 5 main public health hospitals and many movable public health centres prepared for the reception of sick pilgrims. The Public Health Directorate attaches special importance to preventive measures concerning pilgrims such as the care of residences, general hygiene, comfortable accommodation and foodstuffs. The Public Health Directorate has also issued an advisory leaflet containing a summary of preventable diseases, their symptoms, prevention and therapeutic health techniques to deal with them as well as ways and means necessary for safeguarding the environment.

3.2. MATERIAL AND METHODS

3.2.1. Clinical and community study

3.2.1.1. Study design

A cross-sectional survey is the description of the patterns of distribution of disease in population. It aims to describe individuals in the population at a particular point of time, in terms of their personal attributes and their history of exposure to suspected causal agents. These data are then examined in relation to the presence or absence of the disease under investigation. This study was carried out in Jeddah city between October 2001 and April 2002.

3.2.1.2. Main objectives and hypothesis

The main objectives of the study presented in this chapter were to evaluate the prevalence and seasonality of *Cryptosporidium* in children with and without diarrhoea.

The primary hypotheses to be tested were:

1- The ELISA test is more sensitive in detecting infection in faeces than the AP and ZN staining methods.
2- The prevalence of cryptosporidiosis in cases and controls differs in winter and spring.
3- *Cryptosporidium* infection is associated with diarrhoea.
4- *Cryptosporidium* infection is associated with other symptoms, for example nausea and vomiting.
5- There are demographic differences between children with and without diarrhoea.

3.2.1.3 Study Population

The study group included children from 0-6 years of age in 2 categories:

- Children from infant nurseries and pre-schools with and without gastroenteritis.
- Children attending a hospital or clinic as inpatients or outpatients with or without gastroenteritis (children brought to the hospital for other illness).

The definition and classification of diarrhoea followed the WHO criteria as follows: Three or more watery stools in 24 hours as diarrhoea; illness duration of up to 14 days as acute, between 15 and 30 days as persistent and more than 30 days as chronic diarrhoea (WHO, 1998). A case of cryptosporidial infection was defined as a patient with *Cryptosporidium* detected by any laboratory methods.

3.2.1.4. Sample Size

The sample size was calculated as 402 with 252 from schools and 150 from hospitals (the hospital population was sampled in two different seasons, winter and spring). This was based on an expected *Cryptosporidium* spp prevalence of 32% in children (0-5 years) with diarrhoea from my previous Master's project which was performed from April-May 2000 (Al-Braiken *et al.*, 2003). This sample size would thus allow establishing the prevalence in Jeddah city with a precision of +/–5 % (95% confidence limits) and 80% power. The sample size was calculated by using the software Win episcop 20.

3.2.1.5. School sampling

The sample sizes required for the school children with and without diarrhoea were 30 and 222 children respectively. The collection of the stool was done between October 2001 and January 2002. A formal letter from the General Presidency for Girls Education was obtained to visit 6 public schools and infant nurseries. Each of the schools were selected randomly. From each school, 5 children with and 37 without symptoms were selected randomly. The purpose of the study was explained to the principal and the teachers at each school. The teachers and infant nursery attendants were asked to list name, age and sex of all the children. Each child was given a number and then tables of random numbers were used to decide which child was to be chosen for the study. The parent's information sheet (Appendix 1) was circulated by the teachers, infant attendants and/or the administration of the schools to the selected children's parents. When parents did not wish their child to be included, the parents of the next eligible child in their

care selected by random number tables was then approached. The questionnaire (Appendix 3) was sent to the parents to fill in after they had given written consent (Appendix 2).

The information sheets, consent letter and questionnaire were translated into Arabic (Appendices 1a, 2a and 3a). Stool samples were collected from each selected child. All parents were notified of the results after the microscopic investigation of the samples and children were referred to their own doctor for treatment if necessary.

3.2.1.6. Hospital sampling

The sample sizes of the ill children with and without diarrhoea were 100 and 50 children respectively. This was sampled twice, one in the high and one in the low cryptosporidiosis transmission times of winter (October 2001-January 2002) and spring (February-April 2002) to define the seasonality of the infection. A formal letter from the Ministry of Health was obtained to ask four major public hospitals in the area to participate in the study. All the hospitals were selected at random, and a total of 25 children with gastro-enteritis and 13 with other illnesses were selected at random from each hospital. The purpose of the study was explained to the head of the hospital and the chairman of the Paediatric Department of each hospital. The study was explained to the children's parents orally (culturally accepted) and/or by giving them the parental information sheet (Appendix 4). The questionnaire (Appendix 6) was given to the children's parents who agreed to participate in the study, to fill in after they had signed a written consent letter (Appendix 5). Stool samples were collected from each selected child.

As with the school sampling, the information sheet, consent letter and the questionnaire were translated into Arabic (Appendices 4a, 5a and 6a). The parents and the doctors who were looking after the child in the hospital were notified of the results after microscopic investigations of the stool samples.

Figure 3.2.One of the hospitals recruited in the study. King Abdul Aziz Public hospital.

3.2.2. Laboratory methods

3.2.2.1. Sample collection

A single faecal sample was collected from each selected child in the study population. The teachers and nurses were provided with plastic containers with a fitting screw top and disposable gloves. They had been instructed in the safe collection of the faecal samples. Sample containers were labeled with the identification number and date of collection. All the samples collected were immediately placed on ice for subsequent processing and examination in the King Fahad Medical Research Centre (KFMRC). A portion of each of the fresh samples was immediately stored at –80 °C to be used later for ELISA and PCR in Liverpool School of Tropical Medicine (LSTM).

3.2.2.2. Concentration method

The remainder of each faecal sample was concentrated by using a commercial kit. The kit used was the Evergreen faecal parasite concentrator (IDG Ltd, Bury, UK). This kit was stored at room temperature until the indicated expiry date. One kit provides sufficient material for 100 tests.

3.2.2.3. Summary of the Evergreen procedure

All the materials were provided in the kit with the exception of 10% formol saline, concentrated ethyl acetate and 0.85% normal saline (0.85% NaCl in distilled water). The purpose of concentrating the faeces was to increase the possibility of finding the parasite in the samples in which they were too scanty to be seen by direct microscopy. The faeces were emulsified by mixing with 10 ml of 10% formol saline in a flat-bottomed tube (provided in the kit). A green filter in a conical tube (provided in the kit also) was screwed on the mixture tube and then the mixture inverted into the filter tube. This filter catches all the faecal debris and allows the parasites to pass through. Fat materials were then dissolved away from the faeces mixture by the addition of 1.5 ml of ethyl acetate followed by a centrifugation step at 13000xg for 4 min. Following this stage the fatty plug and

the supernatant were discarded and the deposit was re-suspended in 0.85% normal saline.

3.2.2.4. Direct smear

Equipment and reagents

Frosted glass slides	(BDH, Laboratory Supplies, England)
Cover slips	(BDH, England)
Plastic pipits	(BDH, England)
NaCl	(Sigma Chemicals, Poole, Dorset, UK)
Potassium iodide	(Sigma Chemicals, UK)
Iodine crystals	(Sigma Chemicals, UK)

Procedure

Direct smears were prepared by mixing a drop of the concentrated faeces sediment with normal saline on a glass microscope slide and covered with a clean cover slip. The examination was done on all areas under the cover slip at x 40 and x100 magnification. On another slide a drop from the same sample was mixed with a drop of Lugol's iodine (potassium iodide l0g was dissolved in 100 ml distilled water then 5 g iodine crystals was added and stored in a dark bottle) and this was used for detailed identification of parasites cysts. The smear was again examined using x40 and x100. Help was provided from a technician in the Medical parasitology department, King Abdulaziz University in screening some of the slides.

3.2.2.5. *Cryptosporidium* investigation

3.2.2.5.1. AP staining method

Equipment and reagents

Frosted glass slides	(BDH, England)
Phenol crystals	(Sigma Chemicals, UK)
Auramin O	(Sigma Chemicals, UK)

HCl	(Sigma Chemicals, UK)
Ethyl alcohol	(Sigma Chemicals, UK)
Potassium permanganate	(Sigma Chemicals, UK)

Leitz fluorescence microscope with 450-490 nm block filters.

Procedure

The method was as described previously by Casemore and Others (1985). Thin smears were made from the stool concentrator and allowed to air-dry overnight. The slides were covered with pre-filtered auramine solution (Phenol crystals 15g were dissolved in 200ml of distilled water and then 1.5 g of Auramin O was added to the bottle and mixed well. The solution mixture was made up to 500ml with distilled water and stored in a cool dark place) and incubated for 10-15 minutes at room temperature, then rinsed with water. The slides were decolorized in 1% acid alcohol (1% HCl in 95% ethyl alcohol v/v) for two minutes and rinsed with water. The slides were counterstained with potassium permanganate solution for 30 seconds and rinsed with water. The slides were allowed to air dry and then examined using x40 objective on a Leitz fluorescence microscope with a camera attached using blue filter and filter block of 450-490nm with x10 eye pieces. All positive slides were brought back to LSTM for confirmation as quality control.

3.2.2.5.2. ZN staining method

Equipment and reagents

Frosted glass slides	(BDH, England)
Cover slips	(BDH, England)
100% methanol	(BDH, England)
Carbol fuchsin	(BDH, England)
0.4% methylene blue	(BDH, England)

Procedure

The method was as previously described by Casemore and Others (1985). Thin smears of the concentrated faecal samples were prepared and dried for at least one

hour on clean frosted microscopic glass slides. The samples were then fixed with 100% methanol for 5 minutes and stained using the modified ZN as follows: slides were immersed in cold concentrated carbolic fuchsin solution and stained for 15 minutes. The smears were then rinsed in tap water for 4 minutes and decolorized with 1% acid alcohol (as mentioned above) for a few seconds and briefly rinsed in tap water for one minute. Then the slides were counterstained with 0.4% methylene blue for 30 seconds, rinsed in tap water for four minutes and air-dried. Slides were then scanned at x40 and confirmed by oil immersion microscopy at x100 magnification. *Cryptosporidium* spp were identified as ovoid-shaped pinkish red, oocysts averaging 5.0 x 4.5μm or larger. All positive slides were brought back to the LSTM for confirmation as quality control.

3.2.2.5.3. ELISA technique

To confirm the positivity or the negativity of *Cryptosporidium* cases and to enhance the success of finding more positive cases, ELISA test was performed on all cases (n=230) and controls (n=322) by using a commercial kit. The kit used was ProSpecT® *Cryptosporidium* Microplate Assay (Alexon-Trend, Ramsey, USA). The kit was stored at 2-8°C until the expiry date. One kit includes sufficient reagents to perform 96 tests.

3.2.2.5.4. Principles of the ProSpecT® procedure

All the reagents were provided in the test kit. The principle of the test is that *Cryptosporidium* specific antigen (CSA), if present in stools, will be captured by the anti-CSA antibodies coated onto the plate. Diluted stool samples (10 % v/v) were added to each well. After washing three times to remove any unbound material, an enzyme conjugate of monoclonal anti-CSA antibodies labeled with horseradish peroxide enzyme (provided in the kit) was added to the plate. In a positive reaction, CSA binds the enzyme to the well. After washing the plate five times the enzyme substrate, Tetramethyl-Benzadine Buffer (TMB) (also provided in the kit) was added. In a positive reaction, the enzyme bound to the well by CSA converted the substrate to a coloured product. Colour development can be detected

visually or spectrophotometrically. In a negative reaction, there is no CSA or an insufficient level of CSA present to bind the enzyme conjugate and no coloured reaction product develops. This method has been validated in numerous studies (Garcia, 2000; Dagan *et al.*, 1999) although false positives have been identified in one study (Parisi & Tierno, 1995).

3.2.2.5.5. Reading of the results

The results of the tests were read within 10 minutes of adding the stop solution (NaOH, provided in the kit) both visually and also with a microplate spectrophotometer set at 450nm. The test was considered valid if the negative control was colourless or the Optical Density (OD) determined with the spectrophotometer was 0.100 units or less. If the OD of the negative control was greater than 0.100 the results are invalid and the test was repeated with careful attention to the wash procedures. The OD for the positive control should be 0.300 or greater after the OD of the negative control is subtracted. If the OD of the positive sample is less than 0.300, the test should be repeated. The OD of the positive reaction should be ≥0.50 after the OD of the negative control has been subtracted. On the other hand the OD of the negative reaction should be <0.050; this is after the OD of the negative control is subtracted. Visual readings were considered positive if any yellow colour was observed in the well and negative if the well was colorless.

3.2.2.6. Electron microscopy

All the diarrhoeal samples (cases) n=230 were examined using an electron microscope for the presence of viruses using the procedure described by Madeley (1997).

3.2.2.7. Eyepiece calibration

To determine the size of the parasites seen in samples, calibration of the microscope eyepiece was required. This was achieved by using an eyepiece micrometer and a calibration slide. The x10, x40, x100 objectives and eyepieces were calibrated for both light and fluorescent microscopy so that comparable measurements could be made.

3.2.2.8. Photography

Colour photographs were produced by using an Olympus microscope with bright field. The film used was 36 professional colour print film. This film was commercially processed using a Kodak approved agent.

3.2.3. Statistical methods and Ethics

3.2.3.1. Statistical analysis

Data were analyzed using the package EPI-INFO 2000 software package (Centers for Disease Control and Prevention, Atlanta, GA). The statistical significance of the different risk factors were tested by, Chi squared test, Odds Ratio, and 95% confidence intervals in uni-variable and multivariable procedures (logistic regression). Results were considered significant if the *P* value was less than 0.05. The agreement between the different diagnostic methods was assessed by the Kappa test.

3.2.3.2. Ethical considerations

Ethical clearance for the study was obtained from the LSTM Research Ethics Committee on 18 May 2001 (Appendix 9). Official permission for the studies were confirmed by the Ministry of Health and the General Presidency of Girl's Education in the Jeddah area, Saudi Arabia. No appropriate Ethical Committee is available in the area.

3.3. RESULTS

A total of 230 subjects with diarrhoea (cases) and 322 without diarrhoea (controls) were enrolled in the study (Table 3.1). The parents of all ill children agreed to participate in the study, as did the parents of the school children. A total of 200 of the samples from cases and 100 of the control samples were from ill children attending in the hospitals as inpatients or/and outpatients. Thirty of the cases and 222 of the controls were from schools and infant nurseries.

Table 3.1. Participant numbers recruited in the hospital and community study

Study	Cases		Controls	
	Winter	Spring	Winter	Spring
Community	30	NA	222	NA
Hospital	100	100	50	50

The cases were significantly younger than the controls ($P< 0.001$). Their mean ages (SD) were 31.6 (21) and 39.8 (21.3) months respectively (Table 3.2).

Table3.2. The age groups distribution in the cases and controls

Age group	Cases n=230	Controls n=322
0-12 months	59 (25.7%)	59(18.3%)
13-24 months	44(19.1%)	37(11.5%)
25-36 months	36(15.7%)	32(9.9%)
37-48 months	32(13.9%)	56(17.4%)
49-60 months	35(15.2%)	90(28%)
61-72 months	24(10.4%)	48(14.9%)

There were no differences in the educational statues of the mothers of the cases and controls, and no differences in the numbers of adults or children in the households. Cases with diarrhoea were more likely to have contact with domestic animals and to have contact with other diarrhoea patients. They were also more likely to have fever vomiting and abdominal cramps (Table 3.3).

Table 3.3. Demographic characteristics of the cases and controls.

Population characteristic	Cases n=230	Controls n=322	χ^2	OR	95% CI	*P*
Age mean (SD) months	31.6 (21)	39.8 (21.3)				
Median months	29	44				
Mode	60	60				
Sex M: F (M/F %)	115:115(50 %)	153:169(47.%				
Contact with another diarrhoea in the household (%)	53(23%)	37 (11.5%)	**13.1**	**2.31**	1.42-3.75	**0.00**
Contact with domestic animals (%)	29 (12.6%)	23 (7.1%)	**24.7**	**1.88**	1.02-3.47	**0.03**
Mean number of adults in the household (SD)	2.6 (1.54)	2.7 (1.55)				
Mean numbers of children in the household (SD)	2.7 (1.63)	2.9 (1.5)				
The mother's education						
High (%)	131 (57%)	213 (66.1%)	4.83	0.68	0.47-0.97	0.09
Intermediate (%)	65 (28.3%)	67 (20.8%)	0.33	0.80	0.99-2.26	0.07
Primary (%)	11 (4.8%)	19 (5.9%)	1.43	1.44	0.35-1.81	0.5
None (%)	23 (10.0%)	23 (7.1%)	4.10	1.50	0.76-2.75	0.2
Mean duration of diarrhoea (SD)	4.13 (1.32)	NA				
No with vomiting (%)	122 (53%)	10 (3.1%)	**183.9**	**35.2**	**17.21-74.3**	**0.00**
No with abdominal cramps (%)	181(78.7.%)	63 (19.6%)	**190**	**15.19**	**9.79-23.6**	**0.00**
No with fever (%)	83(36.1%)	61(18.9%)	**20.45**	**2.42**	**1.61-3.63**	**0.00**

There was no differences in the ages of cases in hospitals or community, or controls in hospitals and community. The differences of education statues of the mother education was similar among cases in the community or hospital, and in the controls in community and hospitals, except that the hospital controls are more likely than community to have clinical symptoms other than diarrhoea (Table 3.4).

Table 3.4. Demographic characteristics of the cases and controls in the clinical and community study.

	Cases		Controls	
Population characteristic	**Hospital n=200**	**Community n=30**	**Hospital n=100**	**Community n=222**
Age mean (SD) months	31.2 (20.7)	32.5(22)	39.8(21.3)	41(22)
Median months	29	31	44	51
Mode	60	60	60	62
Sex M: F (M/F %)	107:93(46)	8:22(3.5)	75:25(23)	78:144(24)
Contact with another diarrhoea in the household (%)	49(24.5)	4(13.3)	25(25)	12(5.4)
Contact with domestic animals (%)	17(8.5)	12(40)	10(10)	13(5.9)
Mean number of adults in the household (SD)	2.7(1.63)	2.6(1.45)	2.7(1.6)	2.9(1.8)
Mean numbers of children in the household (SD)	2.6(1.54)	2.3(1.2)	2.8(1.5)	2.3(1.7)
The mother's education				
High (%)	110(55)	21(70)	50(50)	163(73.4)
Intermediate (%)	61(30.5)	5(16.7)	39(39)	28(12.6)
Primary (%)	9(4.5)	1(3.3)	2(2)	17(7.7)
None (%)	20(10)	3(10)	9(9)	14(6.3)
Mean duration of diarrhoea (SD)	4.13(1.3)	4(1.2)	NA	NA
No with vomiting (%)	120(60)	2(6.5)	8(8)	2(0.9)
No with abdominal cramps (%)	161(80.5)	20(66.7)	15(15)	48(21.6)
No with fever (%)	79(39.5)	4(13.3)	54(54)	7(3.2)

3.3.1. Comparison of methods of diagnosis

Cryptosporidium oocysts were detected in 15/230 (6.5%) of the cases and in none of the controls by using the ZN and AP staining methods. With the ELISA kit, oocysts were detected in 22/230 (9.6%) and 2/322 (0.6%) of the stools from cases and controls respectively (all from hospital patients). These included all the 15 cases detected with the ZN and AP staining techniques (Table 3.5). That the extent of colour developed in a positive test sample by ELISA kit as visually observed or measured by spectrophotometer seems to correlate with the number of oocysts present in the stool observed with ZN and AP staining methods on that sample but this was not systematically recorded.

Table 3.5. Comparison of the diagnostic methods for *Cryptosporidium.*

Method	Cases n=230		Control n=322	
	Positive N (%)	Negative N (%)	Positive N (%)	Negative N (%)
AP	15 (6.5%)	215 (93.5%)	0 (0%)	322 (100%)
ZN	15 (6.5%)	215 (93.5%)	0 (0%)	322 (100%)
ELISA	22 (9.6%)	208 (90.4%)	2 (0.6%)	320 (99.4%)

There was a good agreement between the different methods in the case group k=0.85. However, in the control group there was chance agreement between the methods used k=0. Table 3.6. Shows the proportional agreement between the diagnostic methods.

Table 3.6. The proportion of agreement between the different diagnostic methods for *Cryptosporidium* (k =0.85 for the cases and k=0 for the controls).

Method	Cases n=230		Control n=322	
	ZN&AP positive	ZN&AP negative	ZN&AP positive	ZN&AP negative
Alexon ProsPecT Positive	15	7	0	2
Negative	0	208	0	320

3.3.2. Laboratory results

The proportion of children with diarrhoea with any intestinal parasites detected in their stool was 120/230 (52.2%) compared with 104/322 (32.3%) among those without diarrhoea. *Cryptosporidium* oocysts were detected among 22 of the 230 (9.6%) children with diarrhoea and 2 of the 322 (0.6%) controls (Table 3.7). The difference in the prevalence rates between the two groups was statistically significantly (P< 0.001, χ^2=25.81, OR=16.92 and 95% CI 3.80-105.24).

Cryptosporidium oocysts (Figure 3.5) were found to be the sole parasite in 21 (95.5%) of the 22 positive diarrhoea cases and in controls. Another coccidian parasite *Cyclospora cayetanensis* (Figure 3.6) was detected in 7 (3%) of the cases and none of the controls. *Giardia lamblia* (Figure 3.8b)(OR 2.6) and *Entamoeba histolytica/dispar* (Figure 3.7*)* (OR 2.44) were also more common in cases than in controls (Table 3.7). Only 1 (4.5%) of the *Cryptosporidium* cases was also associated with *Giardia lamblia* ($P<0.001$).

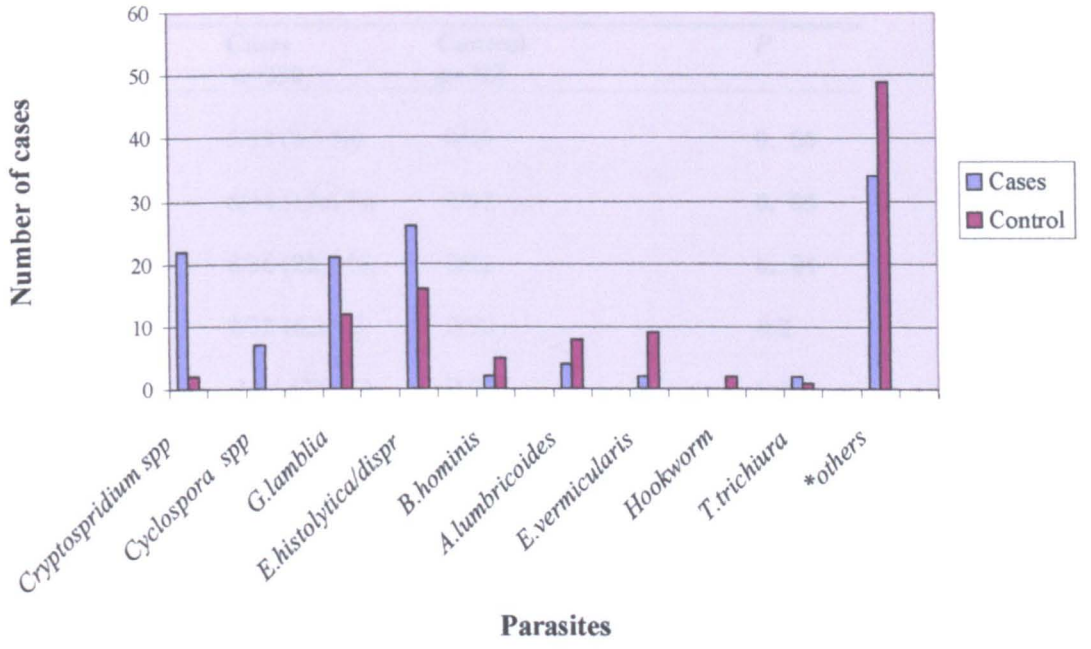
There were low prevalence rates of other parasites (Figure 3.9) in both cases and controls, with no significant differences. Table 3.7 shows the distribution of parasitic infection identified in the cases and controls. The statistical significant ($P< 0.05$) are shown in boldface type.

Table 3.7. Prevalence of various parasites identified in all cases and controls from the study.

Parasites	Cases n=230	Control n=322	χ^2	OR	95 % CI	*P*
Cryptospridium spp	22 (9.6)	2 (0.6)	25.81	16.92	3.80-105.3	**0. 00**
Cyclospora spp	7 (3)	0 (0.)	9.93	NA	NA	**0. 00**
G.lamblia	21 (9.1)	12 (3.7)	6.04	2.60	1.19-5.74	**0. 01**
E.histolytica/dispr	26 (11.3)	16 (5)	6.79	2.44	1.22-4.89	**0. 00**
B.hominis	2 (0.9)	5 (1.6)	0.10	0.56	0.07-3.25	0.7
A.lumbricoides	4 (1.7)	8 (2.5)	0.35	0.69	0.17-2.58	0.5
E.vermicularis	2 (0.9)	9 (2.8)	2.55	0.31	0.05-1.53	0.19
Hookworm	0 (0)	2 (0.6)	0.23	0.00	0.00-5.69	0.6
T.trichiura	2 (0.9)	1 (0.3)	0.09	2.82	0.20-78.9	0.7
*Others	34 (14.8)	49 (15.2)	0.00	0.97	0.59-1.59	0.9

* Other species include *Iodamoeba buetschlii, Chilomastix mesnili, Entamoeba coli, Endolimax nana.*

Figure 3.3. Parasites identified in the study population



There were significant differences in the prevalence rates of *Cryptosporidium* in the various age groups among the cases; there was an increased rate of the infection among the 13-36 months age group. In the controls both *Cryptosporidium* positive cases were found in the 61-72 months age group. The proportion of children with *Cryptosporidium* by age is as shown in Table 3.8 and Figure 3.4.

Table 3.8. The prevalence of *Cryptosporidium* infection by age group in the cases and controls.

Age group	Cases n=230	Control n=322	*P*
0-12	5/59 (8.5 %)	0/59	0. 06
13-24	6/44 (13.6 %)	0/37	**0. 05**
25-36	8/36 (22.2 %)	0/32	**0. 01**
37-48	2/32 (6.3 %)	0/56	0.2
49-60	1/35 (2.9 %)	0/90	0.6
61-72	0	2/48 (4.2 %)	0.7

Figure 3.8. Shows the *Cryptosporidium* infections in each age group in the cases and controls. Age group 1 = 0-12 months, 2 = 13-24 months, 3=25-36 months, 4 = 37-48 months, 5 = 49-60 months, 6=61-72 months.

Figure 3.4. The prevalence of *Cryotosporidium* infection by age group

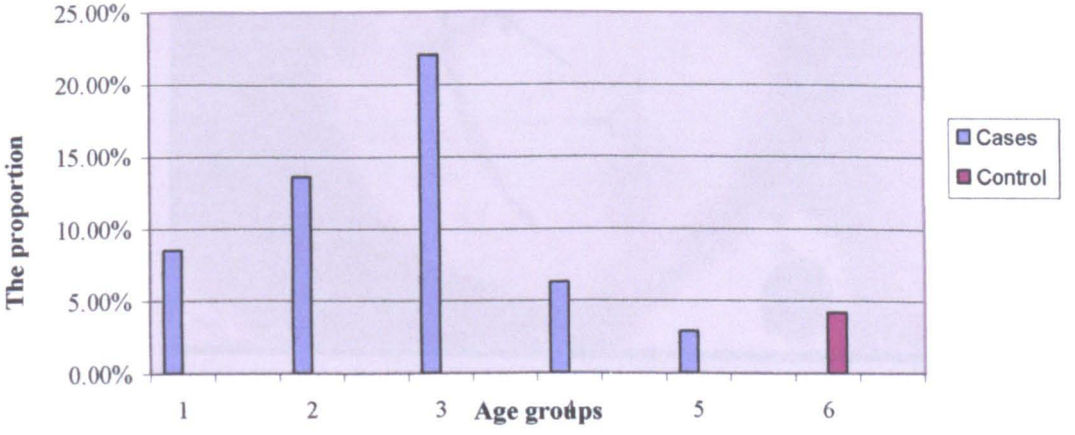
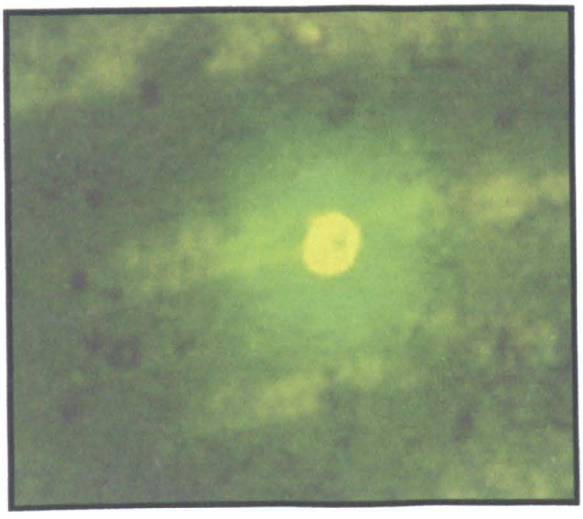
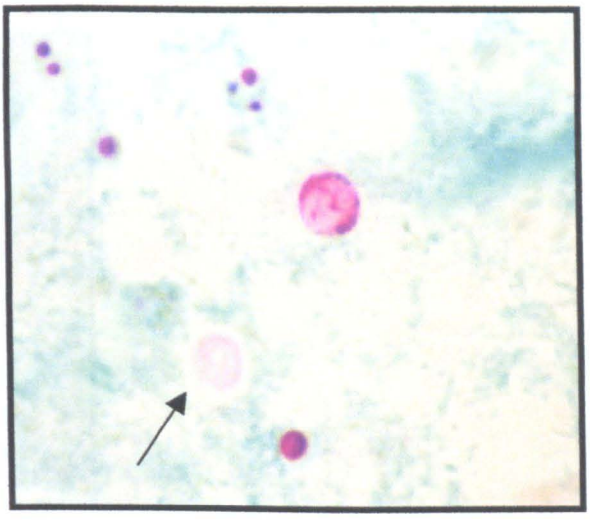


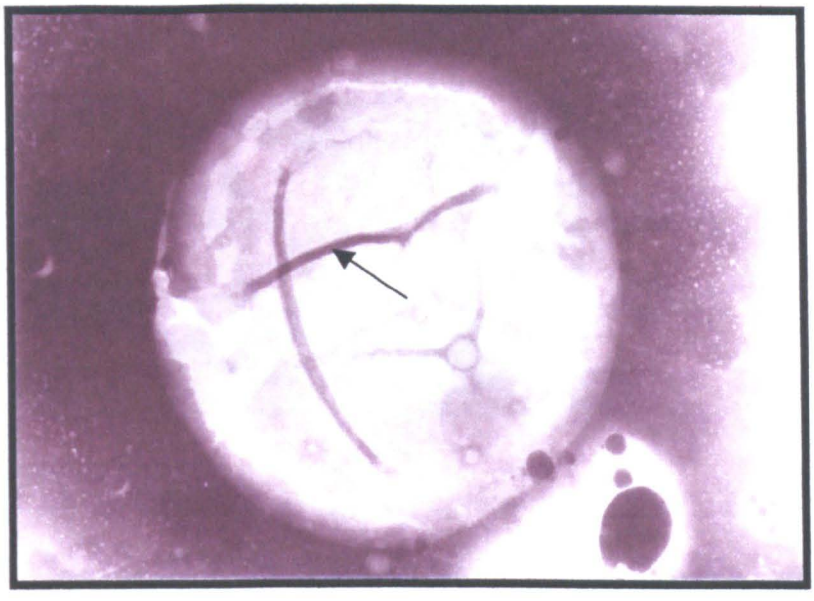
Figure 3.5. *Cryptosporidium* spp from Saudi children.



a



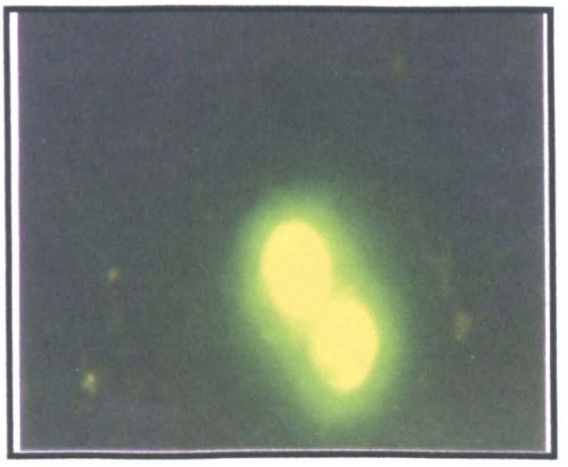
b



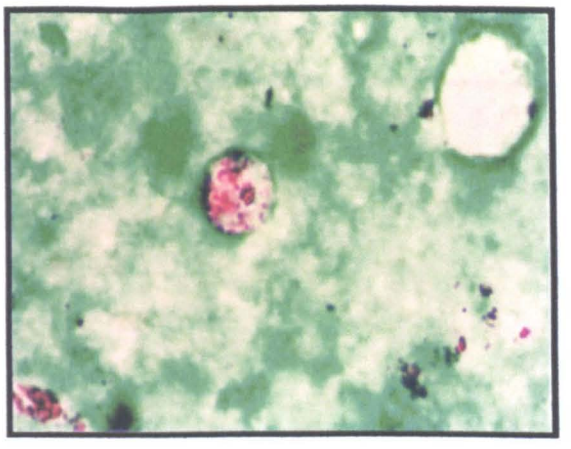
c

Figure 3.5 (a) *Cryptosporidium* oocyst stained with Auramine phenol (1.7×10^3 magnification) (b) stained with Ziehl Neelsen (1.7×10^3 magnification), the arrow shows the ghost oocyst (C) with electron microscopy (14×10^3 magnification), the arrow shows the sature.

Figure 3.6. *Cyclospora* spp identified in the study population.



a

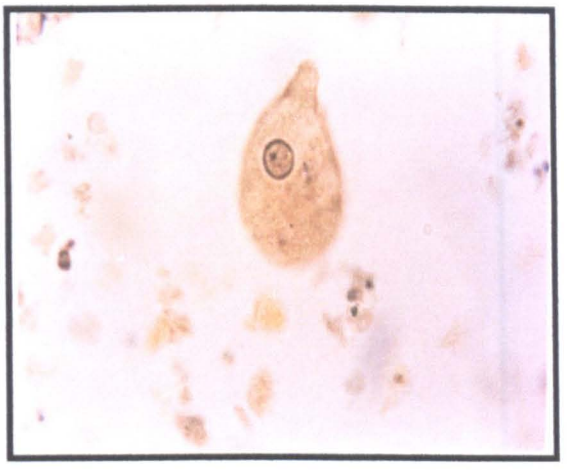


b

Figure 3.6 (a) Shows *Cyclospora* oocyst stained with Auramine phenol (1.4 x 10^3 magnification) (b) with Ziehl Neelsn (1.4 x 10^3 magnifications).

Figure 3.7. *Entamoeba histolytica/dispar* isolates identified in the study population.

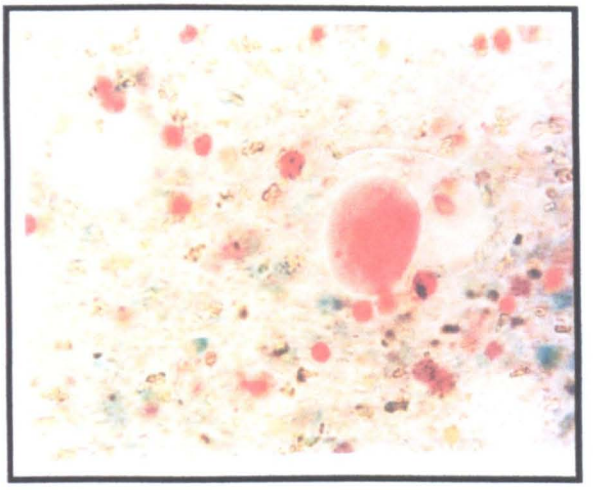
a



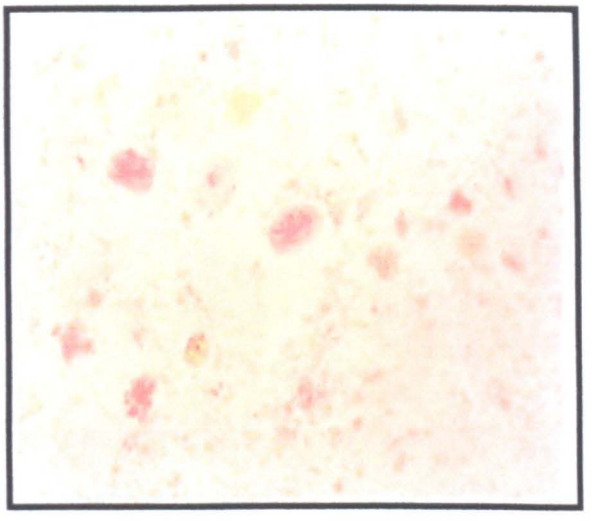
b

Figure 3.7 (a) Shows the cyst of *E.histolytica/dispar* stained with iodine(1.2×10^3 magnification) (b) the trophozoite stage with iodine(1.1×10^3 magnification).

Figure 3.8. Intestinal protozoan identified in the study population.



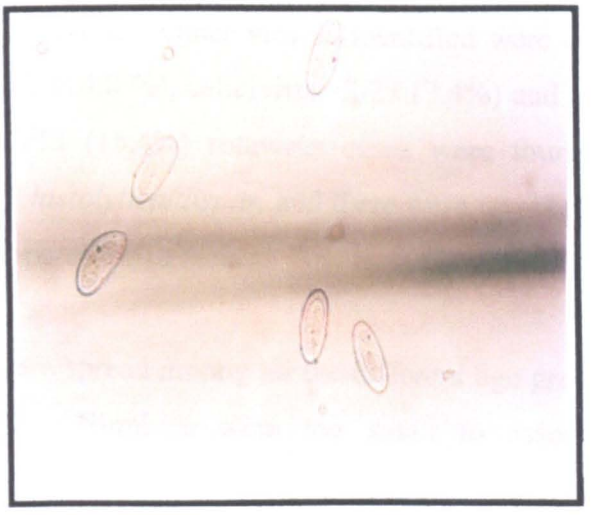
a



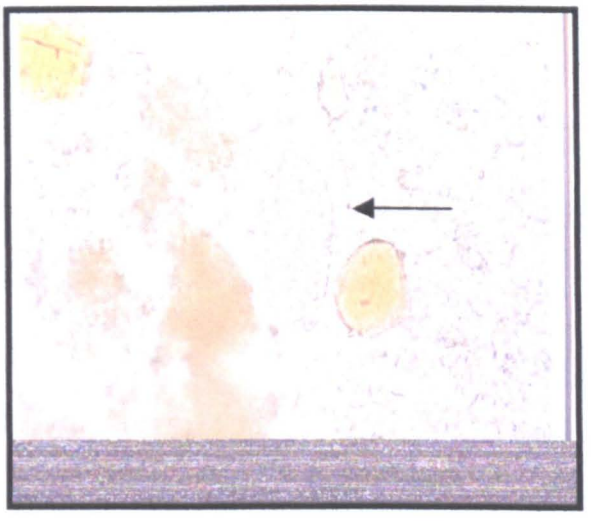
b

Figure 3.8(a) *E.coli* cyst stained with iodine (0.5 x 10^3 magnification) (b) *G.lamblia* cyst stained with iodine (1.1 x 10^3 magnification).

Figure 3.9. Helminth parasites identified in the study population.



a



b

Figure 3.7. (a) Shows *E.vermicularies* egg (0.17×10^3 magnification) (b) Hookworm egg (0.4×10^3 magnification) stained with iodaine stain.

3.3.3. Viral infection

Viruses were detected in 27/230 (11.7%) of the cases (Figure 3.10), of which 13/27 (48.1%) was rotavirus. Other viruses identified were coronavirus 7 /27 (25.9%), adenovirus 4/27 (14.8 %), calicivirus 2/27 (7.4%) and astrovirus 1/27(3.7) (Table 3.9). Only 2/13 (15.4%) rotavirus cases were found mixed with pathogenic protozoa, all *E.histolytica/dispar,* and there were no other cases positive for viruses who had other parasites in faeces.

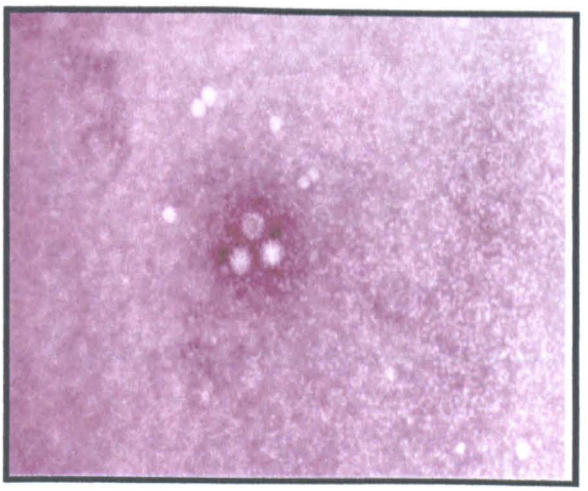
Rotaviruses were spread among all the different age groups, with prevalence from 5.1% to 12%. Numbers were too small to assess statistical significance. Coronaviruses and adenoviruses were found in all age groups but caliciviruses were only found in cases aged 24 months or less.

Table 3.9. The distribution of the viruses identified in the study population by age group.

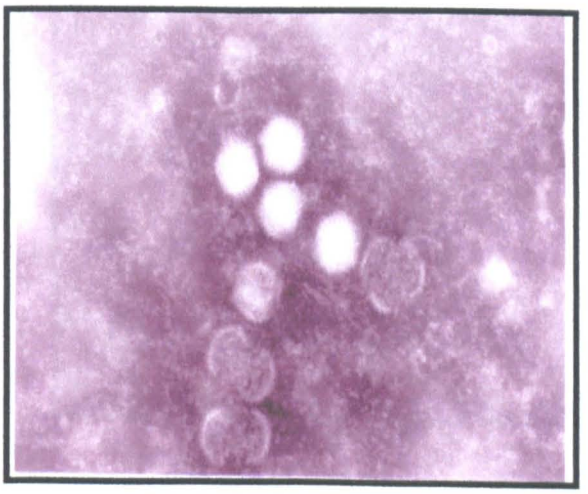
Age group (N)	Rotavirus N (%)	Coronavirus N (%)	Adenovirus N (%)	Calicivirus N (%)	Astrovirus N (%)
0-12 (59)	3 (5.1)	2 (3.4)	1 (1.7)	1 (1.7)	0 (0)
13-24(44)	3 (6.8)	1 (2.3)	1 (2.3)	1 (2.3)	0 (0)
25-36(36)	2 (5.6)	2 (5.6)	0 (0)	0 (0)	0 (0)
37-48(32)	3 (9.4)	0 (0)	1 (3.1)	0 (0)	0 (0)
49-60(35)	2 (5.7)	1 (2.9)	1 (2.9)	0 (0)	0 (0)
61-72(24)	0 (0)	1 (4.2)	0 (0)	0 (0)	1 (4.2)
Total 230	13(5.7)	7(3%)	4(1.7)	2(0.9)	1(0.4)

Figure 3.10. Viruses identified in the study population.

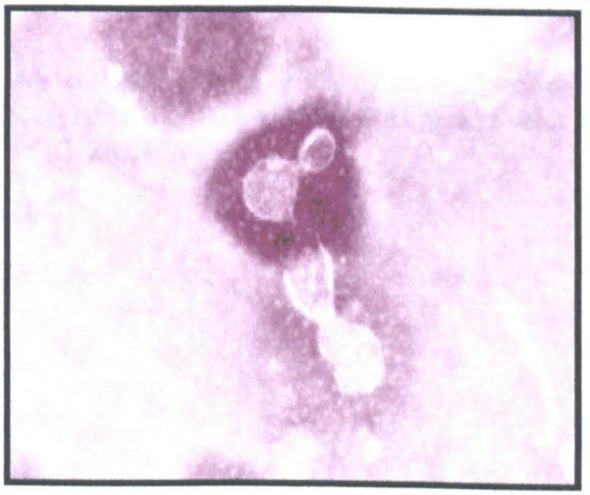
a



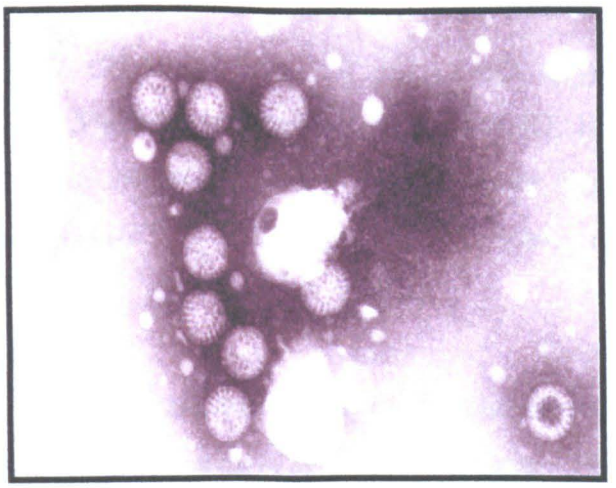
b



c



d



e

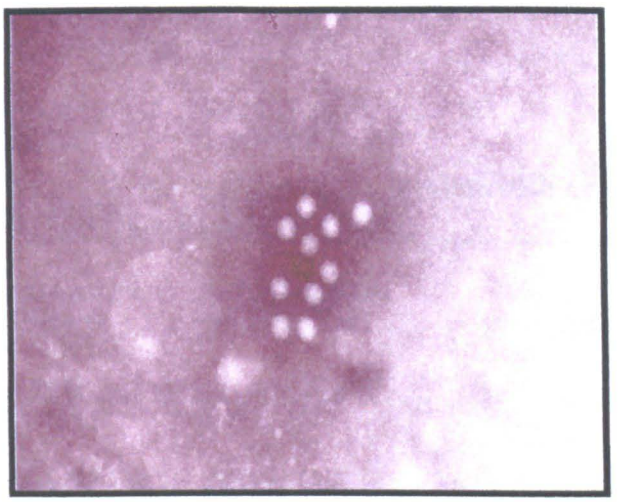


Figure 3.10. Shows (a)calicivirus (1.3 x 10^6 magnification) (b) Adenoviruse (1.2 x 10^6 (c)Coronaviruse(1.1 x 10^6 magnification) (d) Rotaviruse 1.2 x 10^6 magnification) (e)Astroviruse (1.5 x 10^6 magnification).

3.3.4. Characteristics symptoms associated with *Cryptosporidium* infection

A total of 21/22 (95.5%) of the 22 cases with *Cryptosporidium* had acute diarrhoea, 1/22 (4.5%) had persistent diarrhoea (WHO, 1998). On the other hand 174 (83.7%) out of the 208 diarrhoea cases without *Cryptosporidium* had acute diarrhoea and 34 (16.3%) had persistent diarrhoea, no patients had chronic diarrhoea.

Cases with cryptosporidiosis were more likely than other diarrhoeal cases to have acute rather than chronic diarrhea, also almost all cases with cryptosporidiosis had both vomiting and abdominal cramps with statistical significance compared to less proportion of the other cases (Table 3.10). Cases with cryptosporidiosis were also more likely to have fever more than other cases.

Table 3.10. Characteristic symptoms associated with *Cryptosporidium* infection in the diarrhoeal cases.

Characte-ristic	*Cryptospo-ridium* infection n=22	Other diarrhea n=208	χ^2	OR	95%CI	*P*
Acute diarrhoea	21 (95.5%)	174 (83.7%)	2.15	4.10	0.55-84.6	0. 1
Persistent diarrhoea	1 (4.5%)	34 (16.3%)	2.15	0.24	0.01-1.81	0.1
Vomiting episodes	21 (95.5%)	101 (48.6%)	**17.57**	22.25	3.09-452	**0. 00**
Fever episodes	18 (81.8%)	65 (31.3%)	**22.06**	9.90	2.99-36.15	**0. 00**
Abdominal cramps	22 (100%)	159 (76.4%)	**6.59**	NA	NA	**0. 00**

3.3.5. Risk factors associated with *Cryptosporidium* infection

Initial analysis was performed to determine the factors that might be associated with *Cryptosporidium* infection. These factors included sex, age, contact with another person suffering from diarrhoea, source of water supply and contact with animals (Table 3.11 and 3.13).

We found that the age group most frequently affected by *Cryptosporidium* infection in the diarrhoeal cases was those aged, 25-36 months (8/22, 36.4%) with high statistical significance (Table 3.11). However, this was not true for the controls. In this group all of the infections (2) were seen in the 61-72 month age group.

The two main factors associated with cryptosporidiosis infections in diarrhoea cases found in the study population were, a history of contact with a person with diarrhoea in the preceding two weeks and the use of tap water as a source of drinking water. Although, a history of contact with a person with diarrhea in the preceding two weeks was the main risk factor in the overall cryptosporidiosis infection. All other factors tested were found not to be significantly associated with *Cryptosporidium* infection and are as shown in the Table 3.11 and 3.13.

Multivariable analysis using logistic regression was also performed on all variables with $P < 0.1$ and this include all the risk factors identified by the uni-variable analysis. The only risk factor found to be associated with the risk of infection in the cases and the overall cryptosporidiosis infection was a history of contact with a person with diarrhea which was just significance ($P=0.05$) in both groups. The results are shown in Table 3.12 and 3.14.

Table 3.11. The risk factors associated with *Cryptosporidium* infection in the diarrhoea cases.

Exposure variable (n)	positive cases n=22	Negative cases n=208	χ^2	OR	95%CI	*P*
Sex						
M (115)	12 (54.5%)	103 (49.5%)	0.20	1.22	0.47-3.22	0.6
F (115)	10 (45.5%)	105 (50.5%)				
Age						
0-12 (59)	5 (22.7%)	54 (26%)	0.15	0.81	0.25-2.49	0.6
13-24 (44)	6 (27.3%)	38 (18.3%)	0.90	1.62	0.53-4.79	0.3
25-36 (36)	8 (36.4 %)	28 (13.5%)	**7.30**	**3.51**	**1.21-10**	**0.00**
37-48 (32)	2 (9.1 %)	30 (14.4%)	0.26	0.68	0.10-3.27	0.6
49-60 (35)	1 (4.5 %)	34 (16.3 %)	2.17	0.24	0.01-1.80	0.1
61-72 (24)	0	24 (11.5%)	2.83	0.00	0.00-1.85	0.09
Contact with diarrhea (53)	10 (45.5%)	43(20.7%)	**6.89**	**3.20**	**1.18-8.60**	**0.00**
Contact with animals (29)	5 (22.7%)	24 (11.5%)	2.26	2.25	0.66-7.32	0.1
Water source						
Tap water (54)	9 (41%)	45 (21.6%)	**4.11**	**2.51**	**1.0-6.78**	**0.04**
Tap water with filter (96)	8 (36.4%)	88 (42.3%)	0.29	0.78	0.28-2.09	0.5
Bottled water (80)	5 (22.7%)	75 (36.1%)	1.56	0.52	0.16-1.58	0.2

Table 3.12. Logestic regression analysis for the risk factors associated with *Cryptosporidium* infection in the diarrhoea cases.

Exposure variable	O.R	95%CI	P
Age groups			
0-24	1.1	0.25-1.5	0.5
25-48	1.22	0.72-2.7	0.4
49-72	1.2	0.5-1.9	0.6
Contact with diarrhoea	**2.81**	**1.2-8.05**	**0.05**
Contact with animals	2.2	0.64-7.7	0.2
Tap water	1.18	0.38-3.65	0.6
Other source of water	1	0.16-1.9	0.9

Table 3.13. The risk factors associated with overall *Cryptosporidium* infection.

Exposure variable (n)	*Cryptosporidium* positive n=24	*Cryptosporidium* negative n=528	χ^2	OR	95%CI	*P*
Sex						
M (268)	13 (54.2%)	255 (48.3%)	0.32	1.27	0.52-3.09	0.5
F (284)	11 (45.8%)	273 (51.7%)				
Age						
0-12 (118)	5 (20.8%)	113 (21.4%)	0.0	0.97	0.31-2.82	0.9
13-24 (81)	6 (25%)	75 (14.2%)	2.14	2.01	0.69-5.60	0.1
25-36 (68)	8 (33 %)	60 (11.4%)	**9.3**	**3.70**	**1.38-9.68**	**0. 00**
37-48 (88)	2 (8.3 %)	86 (16.3%)	1.08	0.47	0.07-2.11	0.2
49-60 (125)	1 (4.2 %)	124(23.5 %)	2.17	0.14	0.01-1.00	0.2
61-72 (72)	2 (8.3%)	70 (13.3%)	0.49	0.59	0.09-2.70	0.4
Contact with diarrhea (90)	10 (41.7%)	80(15.2%)	**9.38**	**3.48**	**1.38-8.65**	**0.00**
Contact with animals (52)	5 (20.8%)	47 (8.9%)	3.83	2.69	0.84-8.12	0.06
Water source						
Tap water (154)	10 (41.7%)	144 (27.3%)	2.3	1.9	0.7-4.6	0.1
Tap water with filter (198)	8 (33.3%)	190 (36%)	2.54	0.50	0.19-1.27	0.1
Bottled water (200)	6(25%)	194 (36.7%)	1.37	0.57	0.20-1.57	0.2

Table 3.14. Logestic regression analysis for the risk factors associated with *Cryptosporidium* infection in the overall cryptosporidiosis infection.

Exposure variable	O.R	95%CI	P
Age groups			
0-24	1.2	0.2-1.5	0.7
25-48	1.4	0.9-2.9	0.3
49-72	1.2	0.1-1.5	0.4
Contact with diarrhoea	**2.9**	**1.3 -8.89**	**0.05**
Contact with animals	1.1	0.59-1.6	0.3
Tap water	1.5	0.28-2.8	0.7
Other source of water	1.2	0.2-1.3	0.8

3.3.6. Seasonality of *Cryptosporidium* infection

Significant differences were found in the overall prevalence of *Cryptosporidium* infection between the two seasons (Winter and Spring) when the collections were performed. Only 5 of the 24 positive isolates were obtained during the first sampling period October 2001 to January 2002, while included all the community and hospital sampling. The other 19 positive isolates were obtained during the spring sampling period February to April 2002 (P=0.00, χ^2=8.88, OR=4.21, 95%CI=1.43-13.27).

These significant differences were also observed in the prevalence of another coccidian parasite, *Cyclospora* spp but this was found only during the second sampling period with 7% prevalence rate in diarrhoea cases in hospital (P=0.00, χ^2=7.17). No seasonal differences were seen in the prevalence of each of the other parasitic infections identified in the study.

Table 3.15. Seasonality of parasites identified in winter and spring.

	Winter			Spring						
Parasites	**Cases (100)**	**Controls (50)**	**Total**	**Cases (100)**	**Controls (50)**	**Total**	χ^2	**OR**	**95% CI**	**P**
Cryptosporidium	5 (5%)	0	5 (3.3)	17 (17%)	2 (4%)	19 (12.7%)	**8.88**	4.21	1.43-13.27	**0.00**
Cyclospora	0	0	0	7 (7)	0	7 (4.7%)	**7.17**	NA	NA	**0.00**
G.lamblia	7 (7%)	3 (6%)	10 (6.7)	9 (9%)	2 (4%)	11 (7.3%)	0.05	1.11	0.42-2.92	0.8
E.histolytica/ dispar	6 (6%)	9 (18%)	15 (10)	3 (3%)	7 (14%)	10 (6.7%)	1.09	0.64	0.26-1.58	0.2
A.lumbricoides	0	2 (4%)	2 (1.3)	1 (1%)	1 (2%)	2 (1.3%)	0.00	1	0.10-10.07	0.6
E.vermicularis	0	4 (8%)	4 (2.7)	1 (1%)	1 (2%)	2 (1.3%)	0.68	0.49	0.06-3.18	0.4
T.trichiura	0	0	0	0	1 (2%)	1 (0.7%)	1	NA	NA	0.3
Others	11 (11%)	8 (16%)	19 (12.7)	6 (6%)	9 (18%)	15 (10%)	0.53	0.77	0.35-1.66	0.4

3.4. DISCUSSION

Oocysts of *Cryptosporidium* spp are among the smallest exogenous stages of the Apicomplexans; therefore, all morphological differences may not be clear at light microscopic level. The first difficulty in proper identification of *Cryptosporidium* is to distinguish oocysts from other small particles in faecal and environmental specimens such as yeasts, moulds, algae and plant debris. The age and storage conditions of the oocysts and the isolation techniques also can affect the shape of the oocysts and its measurement. Because most oocysts measure 4-6μm, are almost spherical and have obscure internal structures, this can make inter-laboratory comparisons very problematic.

A number of staining methods may be used to assist in identification. These include Ziehl Neelsen (Casemore *et al.*, 1985), Auramine Phenol (Casemore *et al.*, 1985), Wright-Giemsa(Tzipori *et al.*, 1980), Safranin-methylene-blue (Baxby *et al.*,1984), Quinacrine (Ungureauu & Dontu, 1992), Kinyoun (Ma, 1988) and fluorescein isothiocyanate conjugated monoclonal antibody (FITC-mab) (McLauchlin *et al.*, 1987). Commercially available fluorescent labelled monoclonal antibodies (Meridian Diagnostics Inc) significantly increase the sensitivity of direct microscopic examinations but such examinations are still labour-intensive if large numbers of samples are being tested.

This study compared 3 diagnostic methods, ZN, and AP staining methods and an ELISA using the Alexon ProSpecT *Cryptosporidium* microplate assay for the detection of *Cryptosporidium* oocysts in feacal samples. The ELISA was found to be more sensitive (100%) than the ZN and AP (62.5%) as has been found in other studies (Dagan *et al.*, 1995; Garcia *et al.*, 2000).

The ZN and AP techniques were found to be more tedious to perform, time consuming both in the preparation and examination of the slides, more difficult in detecting the oocysts especially in the asymptomatic infections or with low number of oocysts, and a fluorescent microscope was needed in the case of the AP procedure. However, the cost of performing the test was lower. Although the

ELISA kit was more expensive, the test was simple and easy to perform in a short time and also very easy to read the results even in the absence of a spectrophotometer. Use of ELISA greatly enhances laboratories ability to rapidly screen large numbers of samples for the presence of *Cryptosporidium* in stool specimens. Overall, the sensitivities of these assays appear to be superior to traditional microscopy and are comparable to those obtained with immunofluorescent microscopy. However, problems with specificity resulting in false positive test results are of concern and have been reported (Dagan *et al.*, 1995; Parisi &Tierno, 1995).

In the present study 123/230 (52.2%) and 104/322 (32.3%) of the overall diarrhoea cases and controls respectively were found to harbour at least one of the intestinal parasites. The parasite prevalences observed differed as expected between the cases and controls. The prevalence of *Cryptosporidium* in cases was 22/230 (9.6%), significantly higher than in the controls without diarrhoea 2/322 (0.6%) (P=0.00), suggesting that it is positively associated with diarrhoea. The clinical features of *Cryptosporidium* diarrhoea are not sufficiently discriminative to make a positive clinical diagnosis (Crawford & Vermund, 1988; Tzipori, 1988a). This is supported by the findings in the present study. Other studies of *Cryptosporidium* have found it to be significantly associated with persistent diarrhoea in children (Newman *et al.*, 1999). Only one of our cases had persistent diarrhoea, and more cases had acute diarrhoea than cases with other causes of diarrhoea. However, vomiting, abdominal cramps and high temperature were more common in patients with cryptosporidiosis than in other patients with diarrhoea.

In general, the prevalence of *Cryptosporidium* has been found to be higher in infants and toddlers than older children (Casemore *et al.*, 1997). This has been attributed to increased immunological susceptibility, eating behaviour and lack of bowel control in this age group. This association was not observed in this and previous studies. The prevalence of *Cryptosporidium* among infants, toddlers and children above 3 years increased with age (Iqbal *et al.*, 2001; Al-Braiken *et al.*, 2003), perhaps because of the endemicity and more exposure to the infection, as it is known that the

immunity against *Cryptosporidium* infection is only partial. Significantly *Cryptosporidium* was found as mixed infection with *Giardia lamblia* only in one case (P=0.00). In a study in the same area *Cryptosporidium* was associated with *Cyclospora* spp in 11% of cases (Al-Braiken *et al.*, 2003) and in a similar study in Pakistan the co-infection rate was 71.4 % (Iqbal *et al.*, 1999).

Our results also indicate that human infection with *Cryptosporidium* spp in Jeddah city, Saudi Arabia appears to peak in the spring. The greatest number of infection (19/24, 79.2%) was seen during the months of February-April 2002. The seasonality of *Cryptosporidium* in children has been described previously (Clavel *et al.*, 1996; Inungu *et al.*, 2000; Iqbal *et al.*, 2001). The peak seasonal occurrence of cryptosporidiosis is typically during the spring and summer months in temperate climates (Moodley *et al.*, 1991; Meinhardt *et al.*, 1996). However, some studies have identified either primary or secondary peaks in autumn and winter (Casemore *et al.*, 1986; Rodriguez- Hernandez *et al.*, 1996). In a 4 year longitudinal study in Brazil the infection rate during the month of April and May was found to be higher (up to16%) than in other month (Newman *et al.*, 1999). The prevalence of 17% in diarrhoea cases found in the current study in the spring are similar to rates of 32% found in my previous study (Al-Braiken *et al.*, 2003) and support the presence of the seasonality.

Several factors could account for seasonal variations in the occurrence of *Cryptosporidium* including factors affecting the number of oocysts present in the environment such as rainfall or agricultural practices, factors affecting oocyst survival such as humidity or temperature, and factors promoting exposure to oocysts such as contact with animals or attendance at child care centres and/or travelling to countries with high incidence. However, the survey period was not long enough to cover an annual cycle for a complete season variation. In most studies the highest numbers of cases have been detected during the rainy season (Katsumata *et al.*, 1998)

The initial analysis of the different variables found that having had contact with a person with diarrhea and the use of tap water as a source of drinking water were the two main factors associated with the *Cryptosporidium* spp infection. Although, the logistic regression multi-variant analysis demonstrated that only having had contact with a person with diarrhea presented a major risk factor for cryptosporidiosis infection (P=0.05) and this was in agreement with other published reports and suggests a major role for person-to-person transmission (Hojlyng *et al.*, 1986; Newman *et al.*, 1994).

Unlike *Cryptosporidium* species, which are readily infectious after excretion, *Cyclospora* spp the second coccidian parasite identified in this study requires sporulation in the environment before becoming infectious. In this study the prevalence rate of this coccidian parasite was 3% and all the infection were found in spring with a prevalence of 7% in cases with diarrhoea in spring, similar to the 11% found previously in the same area (Al-Braiken *et al.*, 2003). This supports seasonality of *Cyclospora* infection but the numbers are too low to draw further conclusions.

The two most dominant species of parasites found throughout the study population were *E.histolytica/dispar* and *G.lamblia* . *E.histolytica/dispar* was identified in 11 % of the cases and 5 % of the controls and this was comparable to what has been reported for this parasite. In Saudi Arabia prevalences of 1.2%-14% have been reported from different cities in the country (Omar *et al.*, 1991a; Al-Madani & Mahfouz, 1995; Al-Braiken *et al.*, 2003). The diagnosis of amoebiasis by microscopic identification of the parasites in stool is insensitive and still problematic in routine diagnostic laboratories. This is because of the failure to distinguish non-pathogenic amoebae such as *E.dispar* and *E.hartmani* which may increase the apparent prevalence of *E.histolytica* infection. This explains the absence of accurate prevalence data on infections with the invasive *E.histolytica* and therefore it will be important to differentiate between the pathogenic and non-pathogenic strains of amoebae. Moreover, *E.histolytica* cysts have been identified in asymptomatic carriers (Tachibana *et al.*, 2000; Zaki *et al.*, 2001).

G.lamblia was found in 9% and 3.7 % of the cases and controls respectively. In the Middle East several studies have been undertaken to determine the prevalence of *G.lamblia* infection (Ahmed & el Hady, 1989; al-Tukhi *et al.*, 1991; Nimri, 2003; Mukhtar, 1995; Mahmud *et al.*, 1995) showing it to be most common protozoan identified in children. However, the prevalence rate detected in this study is even less than that previously reported for this parasite in Jeddah city, Saudi Arabia (Omar *et al.*, 1991a; Amin, 1997; Al-Braiken *et al.*, 2003). The lack of detection of cases of *G.lamblia* infection in the children during the whole duration of the study may indicate the absence of the infection or the sporadic excretion of the cysts as the cysts can be passed at 2 to 3 or even 7 to 8 day intervals and sometimes the parasites cannot be found in the stools of the patients with giardiasis even with repeated microscopic examination.

In our study the prevalences of *B.hominis* were 0.9% and 1.6% in the cases and controls respectively. Current reports suggest that the parasite is associated with some gut pathology and modulates immune responses indicating it should be considered an opportunistic parasite (Gassama *et al.*, 2001) and, moreover, is significantly associated with the occurrence of watery diarrhoea (Chunge *et al.*, 1995). Our results show that *B.hominis* occurs occasionally among the parasites fauna and this was in agreement to that reported for this parasite in the area (Amin.1997).

The proportion of cases with *Cryptosporidium* (9.6 %) was higher than that of rotavirus (5.6 %). This is comparable to what has been reported elsewhere (Guerrant *et al.*, 1990; Current &Garcia, 1991; Geyer *et al.*, 1993, Henry *et al.*, 1995; O'Donoghe, 1995; Iqbal *et al.*, 1999). Infection with *A.lumbricoides*, *E.vermicularis*, and hookworm were higher in the control group. Moreover, most of these infections were from school children who are deemed to be at higher risk of intestinal parasites.

In this chapter, the ELISA positivity has been taken as the 'gold standard'. Moreover, if we assumed that light microscopy to be the 'gold standard' the conclusion of the analysis of risk factors remain unchanged.

In conclusion, *Cryptosporidium* was amongst the top 2 parasites identified in this study, giving credence to its status as a common cause of childhood diarrhoea. The results indicate seasonal variation of the infection, while is more common in spring. Risk factors of cryptosporidiosis include exposure to other cases of diarrhea and drinking unfiltered tap water whether ELISA results or microscopy alone are to be used to identify cases of infection. Copro-antigen detection of ELISA is more sensitive than microscopy after staining with ZN and AP, assuming that all cases detected by ELISA are true infection. This is explained in chapter 4, with using molecular analysis methods to indicate the presence of *Cryptosporidium* in all positive samples. The results also provide some clues to the general pattern of all aetiological agents of intestinal parasitic disease.

CHAPTER FOUR

MULTILOCUS GENETIC ANALYSIS OF *CRYPTOSPORIDIUM* ISOLATES

4.1. INTRODUCTION

Multilocus analysis (the characterization of a number of different loci (genes) for *Cryptosporidium* spp was undertaken to evaluate the amount of genetic variation within *Cryptosporidium* populations. Moreover, this technique has the potential to better define the strain variations of *Crypotosporidum* spp and to assess the degree of genetic diversity of *Cryptosporidium parvum* cattle genotype (known as genotype 2 and which has retained the name *C.parvum*) and *C.hominis* (the name that has been proposed for the human type (genotype 1) of *C.parvum* (Morgan-Ryan *et al.*, 2002). The aims of this chapter are to examine the possibility to amplify the DNA from all *Cryptosporidium* positive samples identified in chapter 3 with the different diagnostic methods and to identify the species and genotypes from each sample. The results presented here are based on analysis of the 18S rRNA, COWP and gp15/45/60 genes.

4.1.1. The 18S rRNA gene

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 ribosomal RNA (rRNA) genes of *Cryptosporidium* spp are organized according to the paradigm first described for *Plasmodium* spp (Rogers *et al.*, 1995) and subsequently seen in two other Apicomplexans, namely; *Babesia*, and *Theileria* (Kibe *et al.*, 1994). In *C.parvum* there are five copies of rDNA unit per haploid genome with internal structure typical of SSU rRNA namely 18S rRNA, 5.8S rRNA and a large subunit rRNA (LSU rRNA) interspersed by two internal transcribed spacers (ITS1 and ITS2) (Figure 4.1). The five rDNA units are dispersed among at least three different chromosomes. There are two structurally distinct types of rDNA unit, types A and B, with marked differences in the internal

transcribed spacer region. However, there are also polymorphic nucleotides in the 18S rRNA, 5.8S rRNA and the large subunit rRNA genes (Le Blancq *et al.*, 1997). There are four copies of the type A rDNA unit and one copy of the type B rDNA unit per haploid genome. The 18S rRNA gene varies according to the species and genotype of *Cryptosporidium*. The 18S rRNA is ~ 1.750 bp in *C.parvum* and related species *C.hominis* with that of being the longest and *C.baileyi* the shortest. The other species of the parasite including *C.muris* and *C.serpentis* have a slightly longer 18S rRNA gene. The 18S rRNA is highly polymorphic within the genus and is always used as target for the identification and differentiation of *Cryptosporidium* at species and genotype levels. Most of the intra-species differences occur in the first half of the gene. *C.hominis* and *C.parvum* differ in four regions of the gene while the latter and *C.wrairi* also differ in another four regions. However, the differences between *C.parvum* and *C.wrairi* at this gene level are not major (Cai *et al.*, 1992).

Figure 4.1. Diagram for the ribosomal RNA organisation in *Cryptosporidium* spp

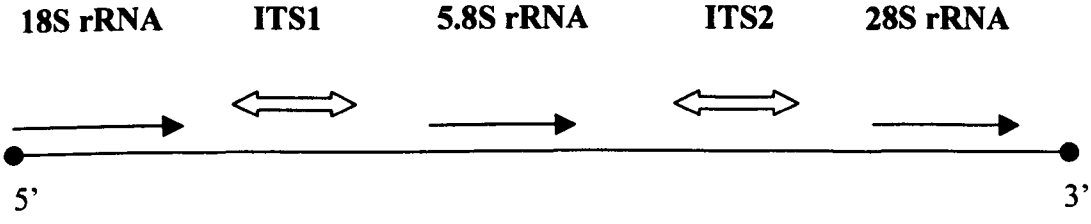


Fig 4.1. Shows the organisation of the ribosomal RNA .The rRNA unit is 5' SSU rRNA, ITS-1, 5.8S rRNA, ITS-2, LSU rRNA 3'.

4.1.2. The COWP gene

This single copy gene encodes a major constituent of the inner layer of the *Cryptosporidium* oocyst wall. Electron microscopy has localized COWP in the wall forming bodies of early and late macrogametes. Ultrastructural analysis of double-walled sporulating and mature oocysts indicated that COWP is selectively localised

in the inner layer of the oocysts. The COWP consists of two large domains both of which contain repetitive amino acid motifs named type I (at the N terminus) and type II (at the C terminus) repeats (Span *et al.*, 1997b). Both repeats contain 6 cysteine residues at conserved and regularly spaced positions and a high proportion of proline and glycine residues. The COWP gene consists of an open reading frame of 4866 bp encoding a polypeptide of 1622 amino acids, which has a molecular mass of 174 Kilodalton (KDa) (Spano *et al.*, 1997a).

The gene coding for COWP is one of the most commonly used targets of genotyping *Cryptosporidium* parasites, because it provides a target unique to *Cryptosporidium* parasites and, thus, it is presumed, has high specificity for all *Cryptosporidium* (Xiao *et al.*, 2000b). However, PCR on the COWP gene fragment 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 fragment analysis (Pedraza-Diaz *et al.*, 2001).

4.1.3. The gp 15/45/60 gene

Two different classes (low and high molecular weight) of antigen have been identified on the surface and apical complexes of the sporozoites and merozoites in previous studies (Ungar & Nash, 1986; Petersen *et al.*, 1992; el-Shewy *et al.*, 1994). The majority of the antigens are present on the zoite apical complex and several are secreted during gliding motility and/or epithelial cell invasion (Ward & Cevallos, 1998). Immunofluorescence microscopy experiments with a number of distinct monoclonal and non-specific polyclonal anti-*C.parvum* antibodies localized seven antigens ranging in size from ~15 to > 1200 KDa to the sporozoite and/or merozoite cell surfaces and at least three (gp 15-17, p23-27 and gp 900) were present in the membranous and proteinaceous trails deposited by sporozoites during gliding locomotion (Strong *et al.*, 2000).

The gp15 /45/60 gene actually encodes a 60 KDa glycoprotein that is processed during intracellular parasite development to produce gp15 and gp45 (Strong *et al.*, 2000). The gp/15 is present on the entire surface sporozoites and merozoites. However the gp/45 present on the apical complex of the sporozoites and on the

apical complex and entire surface of the merozoites (Cevallos *et al.*, 2000), both of which are implicated in zoite attachment to and invasion of enterocytes.

The gp15/45/60 gene is a single copy gene and lacks introns and there is no similarity in the deduced amino acids sequences between gp45 and gp15 (O'Connor *et al.*, 2002). This gene has a high sequence polymorphism particularly among *C.hominis* isolates. There are at least seven alleles for *C.parvum* and *C.hominis* species at this locus and some alleles even exhibited different sub-genotypes (Alves *et al.*, 2003).

4.2. MATERIALS AND METHODS

4.2.1. Samples and their sources

A total of 35 confirmed *Cryptosporidium* positive samples were used for genetic typing. A total of 24 positive samples were collected from the clinics survey, of these 15 samples were identified by using an Acid fast stain; ZN staining technique and AP fluorescent method. This was confirmed also by using a commercial ELISA (Alexon-Trend, USA) and 9 positive samples were identified by ELISA only.

A further 11 samples confirmed to be positive for *Cryptosporidium* by ZN stain only were supplied from King Abdulaziz Hospital in a second visit to the area between November-January 2002-2003.

4.2.2. DNA Extraction

All the faecal specimens were stored unpreserved at –80°C. DNA was extracted by using a commercial kit. The kit used was the QIAMP DNA extraction kit for stool DNA (QIAGEN Ltd, Crawley, West Sussex, UK) according to the manufacturer's instructions. All the reagents were provided in the kit with the exception of 100% ethanol. The kit was stored at room temperature until the indicated expiry date.

4.2.2.1. Oocysts Rupture

To enhance the success of DNA extraction from oocysts of *Cryptosporidium* in stool samples ASL lysis buffer provided in the Q1AGEN kit was added to each eppendorf sample tube to lyse the *Cryptosporidium* oocysts during the freeze/thaw process described below.

Procedure

1- A pea-sized sample of frozen stool sample was scraped into a 1.5 ml eppendorf tube.

2- Approximately 500µl of ASL lysis buffer was added to each tube before allowing the sample to thaw (otherwise the DNA in the sample might degrade).

3- The samples were mixed by vortexing for 30 seconds until they were completely emulsified and homogeneous.

4- The tubes were then subjected to 7 freeze and thaw cycles (starting by heating the samples for 15 min at 80°C then freezing them for 30 min at -80°C) as described by Kim and Others (1992).

4.2.2.2. Q1AMP DNA Extraction kit

Following the final freeze-thaw cycles, an additional 800µl ASL lysis buffer was added into the stool mixture and the tubes were heated up to 70°C for 5 min. Large particles of faecal debris were then pelleted by centrifugation at 13.000 xg for 1 min and 1.2 ml of each supernatant containing proteins and nucleic acids were recovered into another tube. Potential PCR inhibitors were then removed from the supernatant by the application of EX- tablet (provided in the kit). After standing at room temperature for 1 min and subsequent centrifugation at 13.000 xg for 3 min, 200 µl of the supernatant was mixed in a new tube with Proteinase K and AL buffer (also provided in the kit) to digest the proteins. After mixing by vortexing, the solution was incubated at 70°C for 10 min then 200µl ethanol (96%) was added. The samples were vortexed again and then applied to QIAMP column tubes (specific membrane tube into a collection tube, also provided in the kit), which were centrifuged at 13.000 xg for 1 min. Digested proteins and excess enzymes are then washed off with by using the AW1 and AW2 buffers (provided

in the kit) while the DNA is bound onto the membrane in the membrane tube. DNA was then eluted from the membrane with High Performance Liquid Chromatography (HPLC) water into a collecting tube and stored at –20°C until further use.

4.2.3. PCR of *Cryptosporidium* DNA

4.2.3.1. Nested PCR for the 18S rRNA gene

Concept

This nested PCR targets a highly polymorphic fragment of the 18S rRNA gene. The method involves amplifying a large fragment of about 1325bp followed by amplification of an internal fragment of about 840bp from the primary product. This fragment has been shown to provide highly specific information for species assignment and genotypic identification of *Cryptosporidium* (Xiao *et al.*, 1999c).

Procedure

Reagents

10X PCR buffer	(Perkin Elmer (PE) Applied Biosystems, Warrington, Cheshire, UK)
2.5mM dNTPs	(PE Applied Biosystems, UK)
25mM $MgCl_2$	(PE Applied Biosystems, UK)
Ampli-Taq DNA polymerase	(PE Applied Biosystems, UK)
HPLC water	(BDH, England)
Bovine Serum Albumin (BSA)	(Sigma Chemicals, UK)

Primers used

Table 4.1. primer sequences and positions on the complete 18S rRNA gene

Primers	Nucleotide positions	Sequences
External		
AL 1687	156-175	'5-TTCTAGAGCTAATACATGCG-3'
AL1691	1455-1475	'5-CCCTAATCCTTCGAAACAGGA-3'
Internal		
AL 1598	193-218	'5-GGAAGGGTTGTATTTATTAGATAAAG-3'
AL 3032	1008-1029	'5-AAGGAGTAAGGAACAACCTCCA-3'

The primers are adopted from Xiao *et al.*, (1999c).

The primer positions are based on the *C.parvum* 18S rRNA (Accession number AF09349 in GenBank from strain HCNV4) Primers were synthesized by Genosys Oligonucleotides, (Sigma Genosys Ltd Pampsford, Cambridgeshire, UK)

PCR Mixture

Primary PCR master mix

Reagents	100µl Reaction volume
10x Perkin Elmer buffer	10µl
2.5 mM dNTPs	10µl (200µm each)
25mM $MgCl_2$	24 µl (6 mM final)
AmpliTaq DNA Polymerase	1.0 µl (2.5U)
Primer AL 1687	5.0µl (100nM)
Primer AL 1691	5.0µl (100nM)
DNA templates	0.5-1.0 µl
1%BSA	1.0 µl
HPLC Water	Top up to final volume of 100 µl

The 1% BSA was added to the PCR mixture to a final concentration of 0.1mg/ml, to increase the robustness of downstream PCR amplification of the DNA templates.

The amplification

The master mixes were prepared in a clean laminar flow cabinet and dispensed into 200 µl PCR eppendorf tubes by using specific pipettes and filter-protected tips. In specifies and separate DNA area, DNA test templates were added to each tube. The samples were vortexed and loaded into a Techne Thermal cycler (Techne, Ltd, Cambridge, UK). Negative and positive controls were included in all PCR reactions.

The PCR Cycling Conditions were as follow

Initial denaturation	94°C for 3 min
	94°C for 45 sec
For 35 Cycles	55°C for 45sec
	72°C for 1 min
Final extension at	72°C for 7 min

Secondary PCR master mix

Reagents	**100µl Reaction volume**
10x Perkin Elmer buffer	10 µl
2.5mM dNTPs	10 µl (200 µm each)
25mM $MgCl_2$	12 µl (3 mM final conc)
AmpliTaq DNA polymerase	1.0 µl (2.5 U)
Primer AL 1598	5.0 µl (200nM each)
Primer AL 3032	5.0 µl (200nM each)
1% BSA	1.0 µl
Primary PCR product	1.5µl
HPLC Water	Top up to a final volume of 100 µl

The major difference for the secondary reaction was the use of 3mM $MgCl_2$ as the final concentration instead of the 6mM $MgCl_2$ in the primary PCR reaction. The preparation and cycling conditions were the same as for the primary PCR.

4.2.3.2. Agarose gel and electrophoresis

Equipments and reagents used

Agarose Ultra Pure	(Life Technologies, Paisley, Scotland)
Ethidium bromide	(Sigma Chemicals, UK)
1Kb DNA ladder	(Life Technologies, Scotland)
Electrophoresis tank	(Horizon11014 Gibco BRL, Life Technologies, Scotland)
Gel Documentation software	(BioRad, Hemel Hempstead, Hertfordshire, UK)
Tris-borate (TBE) buffer	(5x TBE buffer, 54gm Tris base, 27.5 gm Orthoboric acid, 20 ml 0.5mM EDTA pH 8.0)

Procedure

The separation of nucleic acids by electrophoretic mobility is used for both analytical and preparative purpose. DNA molecules are negatively charged because of their phosphate backbone and the polymer structure results in a constant charge to mass ratio. Therefore in a uniform electric field, nucleic acids migrate through a solid support matrix toward the positively charged anode at a rate that is inversely proportional to the $\log_{10}$ of the molecular weight.

A 2% liquid agarose gel was prepared by weighting 2gm of agarose powder and adding it to 100ml of 0.5x TBE buffer. The mixture was heated to boiling in a microwave oven for approximately 5min to produce homogenous mixture. Then 4µl of (0.05%w/v) of ethidium bromide was added to the mixture after it had cooled for 5 min. It was then mixed thoroughly and poured into the plexiglas gel support system with the comb positioned. The mixture was allowed to cool resulting in the formation of a horizontal slab gel containing slots at one end for sample loading. The gel support system was then placed in electrophoresis tank and 0.5x TBE poured in to a level sufficient to cover the gel.

The 2µl of loading buffer (containing bromophenol blue 0.05%w/v) was mixed with 8µl of the secondary PCR products and this was loaded into the wells. Negative and positive controls were also included in each run.

A 120-volt electric current was applied to separate the samples by electrophoresis. The power was stopped when the bromophenol blue bands had migrated to the edge of the gel at the positive anode. The DNA bands were visualized by using ultra-violet trans-illumination and by using the gel documentation system photographs were taken. The DNA bands for each sample were sized according to the migration pattern of the standard DNA ladder.

4.2.4. RFLP for the 18S rRNA

Concept

Restriction endonucleases are enzymes that bind to specific DNA sequences within a DNA chain and create double strands of DNA broken by enzymatic cleavage. The recognition sites are usually of about 4-8 bp and these restriction enzyme recognition sites are highly specific to each enzyme. It is possible to use commercially available enzymes.

Two different endonuclease enzymes *Ssp*I and *Vsp*I were used for this as they help in the differentiation between *Cryptosporidium* species and divide them into genotypes as described previously by Xiao and Colleagues (1999c). In addition, some of the non- parvum *Cryptosporidium* species have a different restriction pattern with *Ssp*I.

Enzymes used

Table 4.2. Restriction endonucleases sources and cleavage positions.

Enzyme	Biological source	Manufacture source	Restriction site
*Ssp*I	*Sphaerotilus* spp	Boehringer ,Mannheim, UK	AAT ↓ ATT TTA ↑ TAA
*Vsp*I	*Arthrobacter* spp	Boehringer, UK	AT ↓TAAT TAAT ↑ TA

The restriction endonucleases reaction

Reaction 1

*Ssp*I 20U	2.0µl
Buffer	5.0µl
Water	23.0µl
2nd PCR product	20.0µl
Total volume	50.0µl

Reaction 2

*Vsp*I 20U	2.0µl
Buffer	5.0µl
Water	23.0µl
2nd PCR product	20.0µl
Total volume	50.0µl

Procedure

1-The mixtures containing the restriction enzymes with the different amplicon samples were incubated at 37°C for one hour.

2-The digested fragments were separated by electrophoresis on 2% agarose gels as described previously. The fragments bands were visualized by ultraviolet trans-illumination.

Table 4.3. Predicted RFLP digestion patterns for *Cryptosporidium* spp based on 18S r RNA gene

Species	Original host	PCR length	*Ssp*I (fragment bp)	*Vsp*I (fragment bp)
C.hominis	Humans	837	449,254,111	561,104,70
C.parvum (genotype 2)	Cattle	834	449,254,108	628,104
C.meleagridis	Turkeys	833	449,254,108	456,171,104
C.muris	Cattle, camel, hyrax	833	448,385	731,102

Adapted from Xiao *et al.*, (1999c). Note only size of visible fragments are shown.

4.2.5. Standard PCR for COWP gene

Concept

A fragment (550bp) of the COWP gene was targeted. This fragment encompasses part of amino acid motif type I. The PCR conditions used were as previously described by Spano and Others (1997b).

Primers used

Table 4.4. Primers sequences and position on the complete COWP gene

Primers	Nucleotide Position	Sequences
Cry -15	921-943	5'-GTAGATAATGGAAGAGATTGTG-3'
Cry -9	1445-1470	5'GGACTGAAATACAGGCATTATCTTG3'

Primers adopted from Spano *et al.*, (1997b).

The primer positions are based on *C.parvum* (Accession number Z22537 in GenBank, from strain MI-ISS-1 Complete COWP gene sequence). Primers were synthesized by Genosys Oligonucleotides (Sigma Genosys Ltd, UK).

PCR Mixture

Reagents	**100µl total volume**
10x Perkin Elmer buffer	10µl
2.5Mm dNTPs	10µl (200µM each)
25mM $MgCl_2$	6µl (1.5mM)
AmpliTaq DNA Polymerase	1µl (2.5U)
Primer Cry-15	6µl (10mM)
Primer Cry-9	6µl (10mM)
1%BSA	1µl (0.1mg/ml)
DNA Test sample	15-20 µl
HPLC water	Top up to a final volume of 100µl

The amplification

As stated in the 18S rRNA method, the master mixtures were prepared in a clean laminar flow cabinet and dispensed into 200µl PCR eppendorf tubes. DNA test samples were added. The samples were vortexed and centrifuged at 13000xg for 30 sec. Then the mixtures were loaded into a thermal cycler.

The amplification condition

Initial denaturation	94°C for 3 min
35 cycles of	94°C for 60sec
	52°C for 30 sec
	72°C for 60 sec
Final extension	72°C for 10 min.

4.2.6. Gel electrophoresis

DNA amplicons in an 8μl volume from the PCR reaction were separated by electrophoresis on a 2% agarose gel as described previously for the 18S rRNA PCR. The amplicon bands were visualized by ultraviolet trans-illumination.

4.2.7. RFLP for COWP gene

The PCR products were subjected to restriction endonuclease digestion by *Rsa*I. The digestion products were separated by agarose gel electrophoresis. The product size was confirmed by comparison with a DNA molecular mass standard marker.

Enzymes used

Table 4.5. Restriction endonuclease source and cleavage position

Enzyme	Biological source	Manufacturer source	Restriction site
*Rsa*I	*Rhodopseudomonas sphaeroides*	Roche Molecular Biochemical, Mannheim ,Germany	GT ↓ AC CA ↑TG

The restriction reaction

Reagent	volume (μl)
*Rsa*I Enzyme (10u/μl)	1.0μl
10x buffer C	2.0μl
Water	7.0μl
PCR reaction	10.0μl
Total volume	20.0μl

Procedure

The mixture containing the restriction endonuclease and the PCR products was incubated at 37°C for a minimum of 3 hours.

The digestion fragments were separated by agarose gel electrophoresis and visualised by ultraviolet trans-illumination.

Table 4.6. Predicted *Rsa*I patterns of COWP PCR products for various *Cryptosporidium* spp

Species (genotypes)	Original host	PCR length	Sizes of RFLP bands (bp)
C.hominis	Human	550 bp	284,129,106
C.parvum (genotype 2)	Cattle	550 bp	413,106
C.meleagridis	Turkey	550 bp	372,147
C.muris	Mice	550 bp	327,104,86

Adapted from Xiao *et al.*, (2000b). Note only sizes of visible fragments are shown.

4.2.8. Standard PCR for gp/15/45/60 gene

1-Kb fragment extending from the translational start codon to the termination codon of the gp/15/45/60 gene was targeted. The amplification conditions used were as previously described by Strong and Others (2000).

Primers used

Table 4.7. primer sequences and positions on the complete gp/15/45/60 gene

Primers	Nucleotide position	Sequence
Gp15ATG	275-306	5-CGGGATCCATATGAGATTGTCGCTCATTATC-3'
GP15STOP	1252-1279	5'-GGAATTCTTACAACACGAATAAGGCTG-3'

Primers adopted from Strong *et al.*, (2000).

The primers positions are based on *C.parvum* (Accession number AF022929 in GenBank from strain N1Ncl). The primers were synthesized by Genosys Oligonucleotides (Sigma, Genosys Ltd, UK).

PCR mixture

Reagents	**100µl total volume**
10x Perkin Elmer buffer	10.0µl
2.5mM dNTPs	10.0µl (200 µm each)
25 mM $MgCl_2$	6.0µl (1.5 mM final)
Amplitaq DNA polymerase	1.0µl (2.5U)
Primer Gp15 ATG	6.0µl (10mM)
Primer Gp 15 stop	6.0µl (10mM)
DNA test sample	15-20 µl
1% BSA	1.0µl
HPLC water	Top up to a final volume of 100µl

The amplification

The mixture was mixed with the DNA test sample and loaded in the thermal cycler.

Condition

Initial denaturation:	95°C for 4min
35 cycles	95°C for 30 sec
	50°C for 45sec
	72°C for 1min
Final extension	72°C for 10 min

The PCR products were separated on a 2% agarose gel and visualised by ultraviolet trans-illumination having stained the gel with ethidium bromide.

4.3. RESULTS

4.3.1. Nested PCR and RFLP for 18S rRNA gene

A total of 35 *Cryptosporidium* positive samples were tested by targeting the 18S rRNA gene, and 31/35 (88 %) of the samples yielded PCR products. There were 4/35 (11%) samples that did not yield any amplicons. The ELISA test was the only that confirmed the presence of *Cryptosporidium* spp in three samples and one sample was positive by both microscopy and ELISA.

The secondary PCR yielded amplicons in the size range of between 833-837 bp depending on the *Cryptosporidium* species and genotype (Table 4.8). This is related to the expected fragment size of the internal target sequence of the 18SrRNA gene for *Cryptosporidium* species and this product was obtained after amplifying the internal fragment from the primary PCR amplicons of about 1325bp in length (Figure 4.2).

These secondary PCR products were then digested by using two different endonuclease *Ssp*I and *Vsp*I to define the different *Cryptosporidium* species and genotype. With the use of *Ssp*I digestion, 3 band patterns characteristic of the presence of *C.parvum*, *C hominis*, and *C.meleagridis* were visible (Figure 4.3). The band patterns were of 449bp, 254 and 108-111 bp (lanes 3-5 and 7-12). In the case of *C.muris*, 448 and 385 bp fragments were present. To differentiate genotypes, an additional digestion with the *Vsp*I restriction endonucleases enzyme was used.

In Figure 4.3 (lower panel) lanes 3,4,7,9 and 11 showed fragments corresponding to *C.parvum* bovine genotype of approximately 628,104 bp. Lanes 5,8 and 12 showed fragments identified as *C.hominis* with fragments size of approximately 561 and 104 bp. In lane 10 fragments of approximately 456, 171, 104 bp indicated the presence of *C.meleagridis*. The largest fragments of approximately 731 and 102 bp were seen in lane 6 which corresponded to the pattern expected for *C.muris*.

C.parvum was detected in 15/35 (42.9%), *C.hominis* in 13/35(37%), *C.muris* in 1/35(2.9%) and *C. meleagridis* was also detected in 1/35 (2.9%) of the patients.

The *Vsp*I restriction profile for one sample (C115) showed multiple fragments of 628,561,104, bp. These sites are likely to be for *C.parvum* bovine genotype (628 and 104 bp) and *C.hominis* (561 and104 bp). To confirm this observation sequence analysis for this sample has been carried out.

Table 4.8. Size of the PCR product and restriction sites for the samples identified in the study based on analysis of the 18S rRNA gene.

Species	PCR product	Fragment size	
		***Ssp*I**	***Vsp*I**
C.parvum n=15	834	449,254,108	628,104
C.hominis n=13	837	449,254,111	561,104
C.meleagridis n=1	833	449,254,108	456,171,104
C.muris n=1	833	448,385	731,102

Table 4.8. Shows the size of the PCR products and the fragment sizes for the samples identified in the study population by using the two enzymes *Ssp*I and *Vsp*I.

Figure 4.2. Results of Nested PCR for *Cryptosporidium* spp for the 18S rRNA

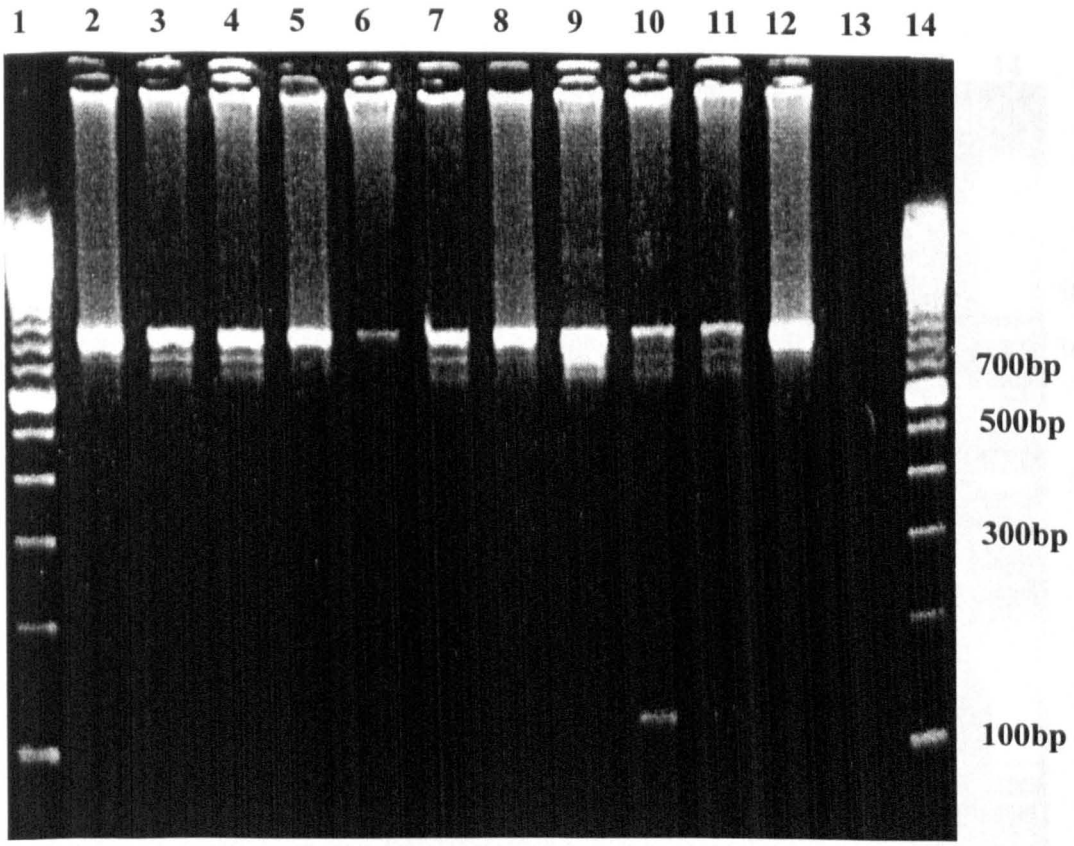
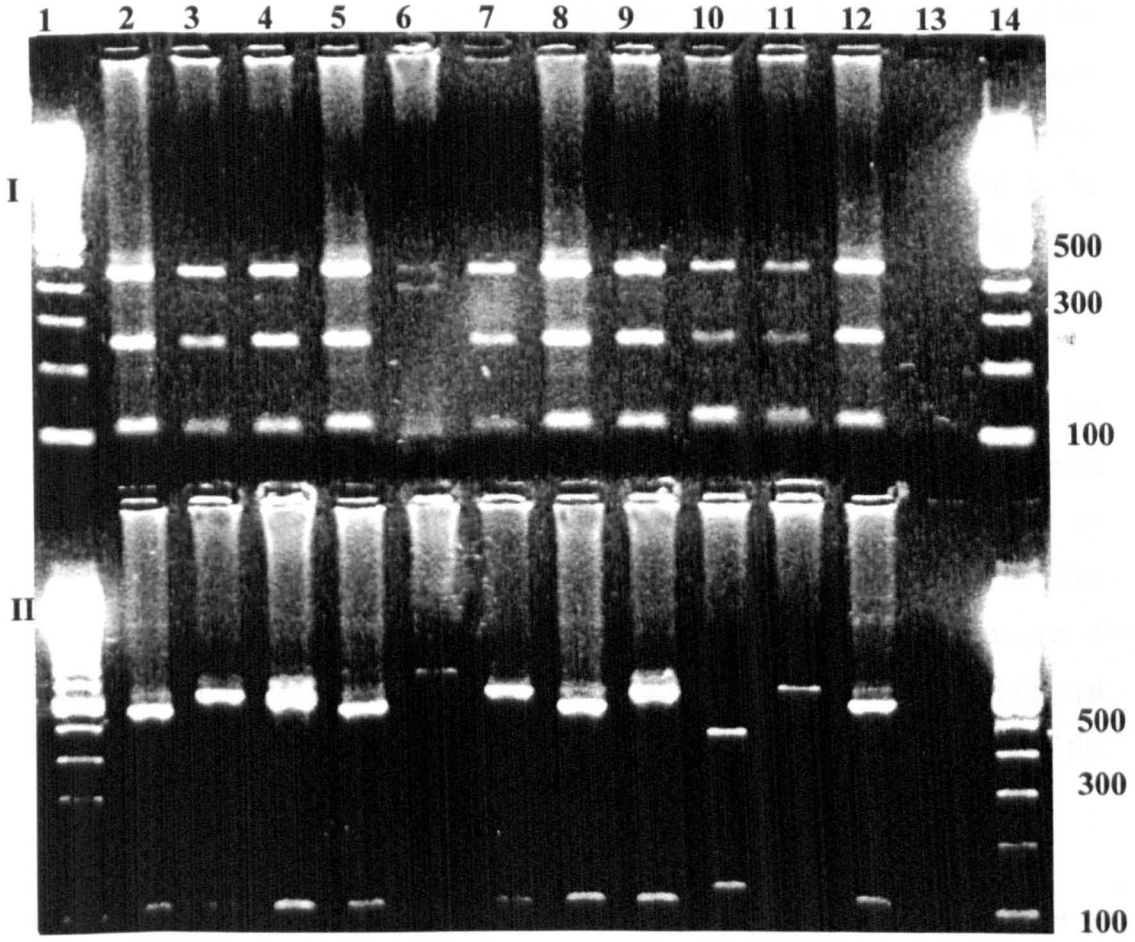


Figure 4.2 Visualisation of PCR products generated after secondary PCR. Lanes 1 and 14 show the 1 Kb molecular weight marker. Lane 2 shows the positive control while lanes 3-12 show the samples from the study. Lane 13 shows the negative control sample

Figure 4.3. Restriction Fragment Length Polymrophism (RFLP) for 18S rRNA



I= *Ssp*I digestion; II= *Vsp*I digestion

Figure 4.3. Visualisation of the restriction endonuclease digestion of the 18S rRNA gene. Panel I shows the results of restriction digestion with *Ssp*I. Lanes 3-5 and 7-12 show *C.parvum*, *C.hominis* and *C.meleagridis*, lane 6 shows *C.muris.* Panel II shows the digestion with *Vsp*I. Lanes 3, 4, 7, 9 and 11 show *C.parvum*; Lanes 5, 8,and 12 show *C.hominis;* .lane 10 shows *C.meleagridis* and lane 6 shows *C.muris*. Lanes 1 and 14 show the 1Kbmarker; lane 2 shows the positive control; lane13 show the negative control

4.3.2. PCR and RFLP for COWP gene

The primers Cry -15and Cry-9 were used to map the N terminal domain of the COWP gene for the 35 *Cryptosporidium* positive samples. 30/35 (85.7%) of the samples yielded a PCR product of approximately 550bp encoding type 1 repeats from residues 141 to 323 (Figure 4.4 and Table 4.9). The four samples for which no products were obtained with the 18S rRNA gene target also failed to be amplified by using this locus. Difficulties were experienced with the PCR amplification of DNA from *C.muris* and we failed to amplify this sample.

The PCR products were digested with the restriction enzyme *Rsa*I. All *Cryptosporidium* (excluding *C.meleagridis)* isolates could be arranged into two distinct groups (Figure 4.5). One group, lanes 3,4,7,9 and 10 was characterized by a 3 band pattern (413 and 106 bp) this comprised the *C.parvum* isolates .The second group (lanes 5 and 11) comprising the *C.hominis* group displayed 4 restriction fragments of 284, 129 and 106 indicating the presence of an additional polymorphic *Rsa*I restriction site splitting the 413 bp fragment characteristic of the animal isolates as stated previously by Spano and Colleagues (1997b).

In lane 6 there was a 3 visible bands pattern 372, and 147 bp and this is characteristic of *C.meleagridis*. As in the 18S rRNA gene target, *C.parvum* was detected in 15/35(42.9) samples, *C.hominis* in 13/35(37%) and *C.meleagridis* in 1/35(2.9%) sample.

Unusual restriction band pattern was observed in lane 8 for sample (C115) at 550,413,284,106 bp, as stated previously for the 18S rRNA gene, this sample may manifest a mixed infection or a novel species; sequence analysis has been carried out for this sample.

Table 4.9. Restriction sites and size of product for the samples identified in the study based on the COWP gene.

Species/genotype	PCR product	Fragment size *Rsa*I
C.parvum n=15	550 bp	413,106
C.hominis n=13	550 bp	284,129,106
C.meleagridis n=1	550 bp	372,147

Table 4.9. Shows the size of the PCR products and the fragment sizes for the samples identified in the study population by using the restriction enzyme *Rsa*I.

Figure 4.4. Results of COWP –PCR for *Cryptosporidium* spp

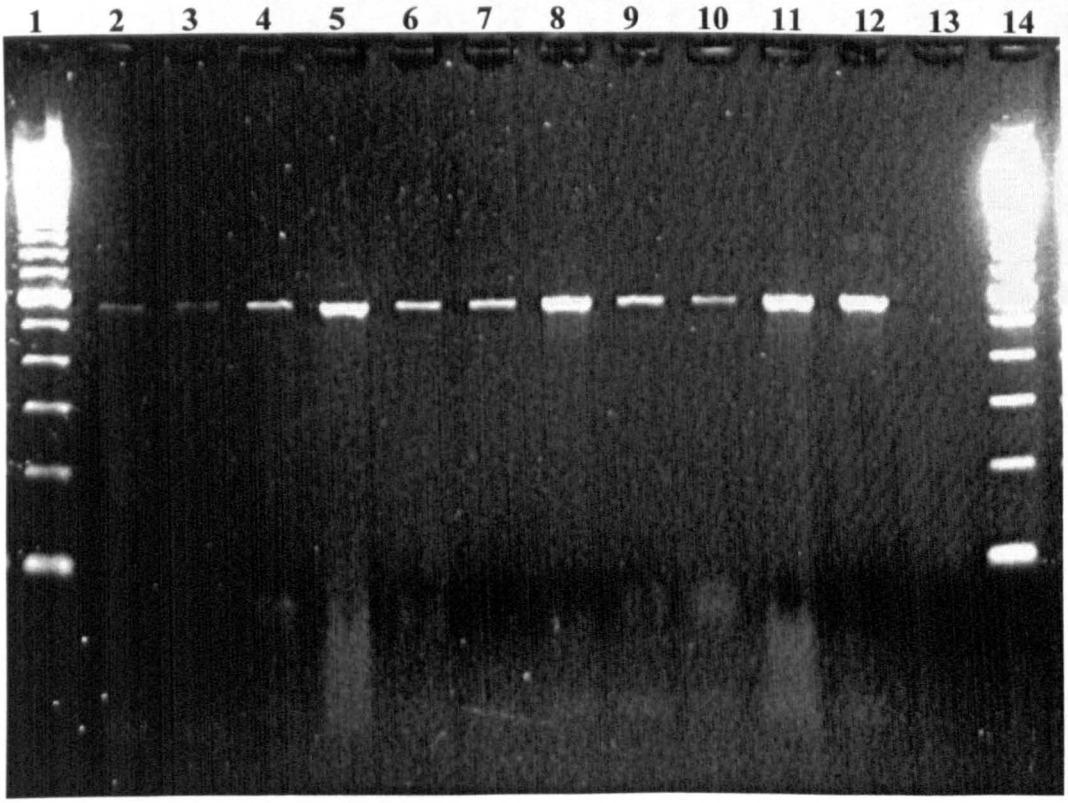


Figure 4.4 Visualization of PCR products generated after COWP gene PCR. Lanes 1 and 14 show the 1Kb molecular weight marker. Lane 2 shows the positive control while lanes 3-12 show the samples from the study . Lane 13 shows the negative control

Figure 4.5. Restriction Fragment Length Polymorphism (RFLP) for COWP gene.

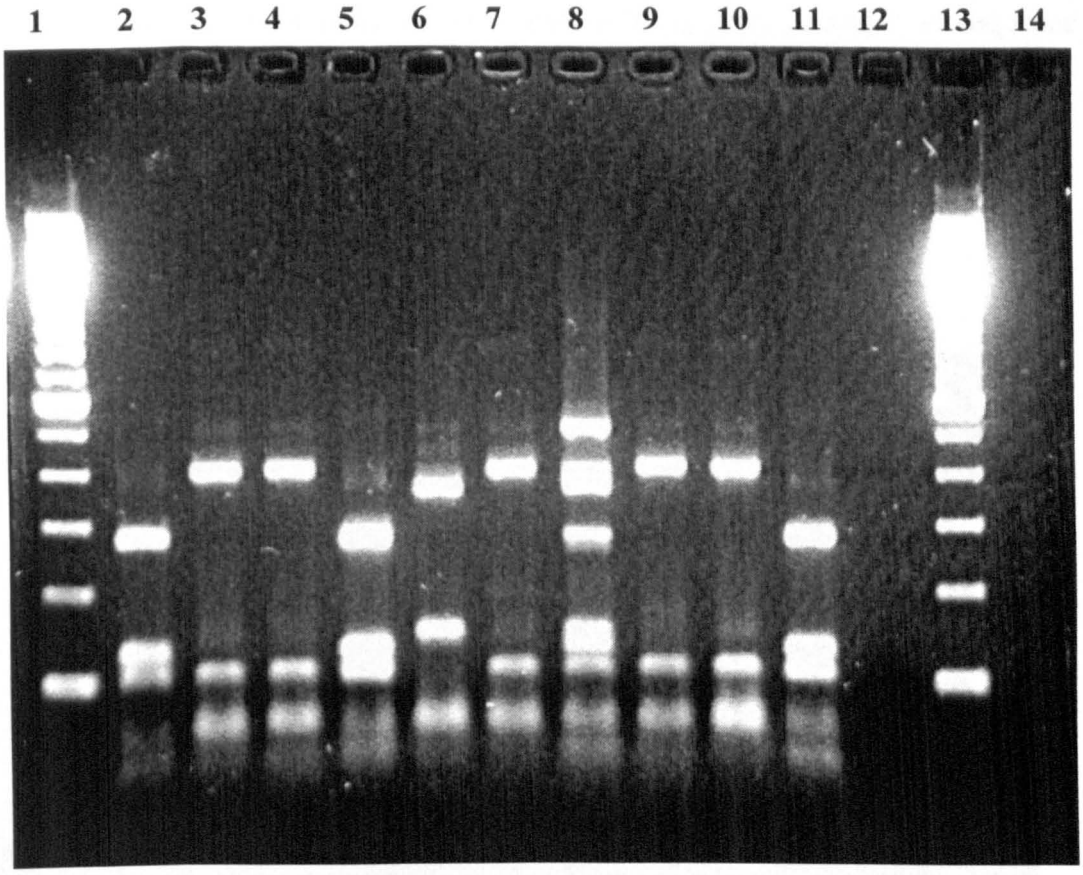


Fig 4.5.Visualisation of restriction endonuclease digestion *Rsa*I of the COWP-gene for *Cryptosporidium* species. Lanes 1 and 13 show the 1Kb molecular weight marker. Lane 2 shows the positive control; lane 12 shows the negative control; Lanes 3, 4, 7, 9, and 10 show *C.parvum*; Lanes 5 and 11 show *C.hominis*; lane 6 show *C.meleagridis* and lane 8 show sample C115

4.3.3. PCR for gp/15/45/60 gene

All *C.parvum* isolates15/35 (42.9%), *C.hominis* 13/35 (37%) and C115 (undefined sample) were amplified by using the primers gp15ATG and gp15stop.

All the samples yielded PCR amplicons of about 1-Kb and a slight differences in the size of PCR products among some of the *C.hominis* isolates were observed as demonstrated in lanes 7 and 12 (Figure 4.6).

Sequence analysis has been undertaken to classify the different alleic groups of *C.hominis* and the bovine genotype of *C.parvum* isolates based on this locus. To clarify *Cryptosporidium* infection in sample (C115) gene cloning and sequence analysis have been carried out (see chapter 5).

Fig 4.6. Results of gp15/45/60 -PCR for *Cryptosporidium* spp

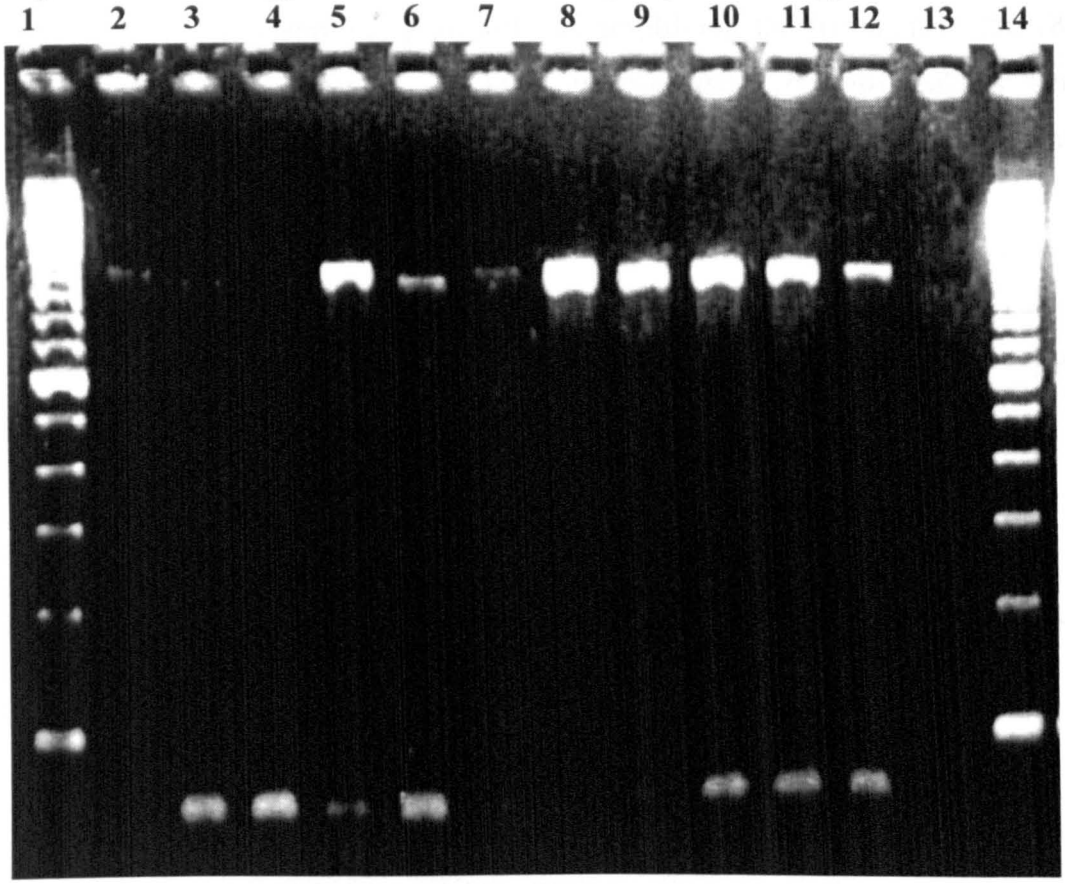


Fig 4.6. Visualization of PCR products generated after gp/15/45/60-PCR Lanes 1 and 14 show the 1Kb molecular weight marker. Lane 2 shows the positive control; lanes 3-12 show the test samples; lane 13 shows the negative control.

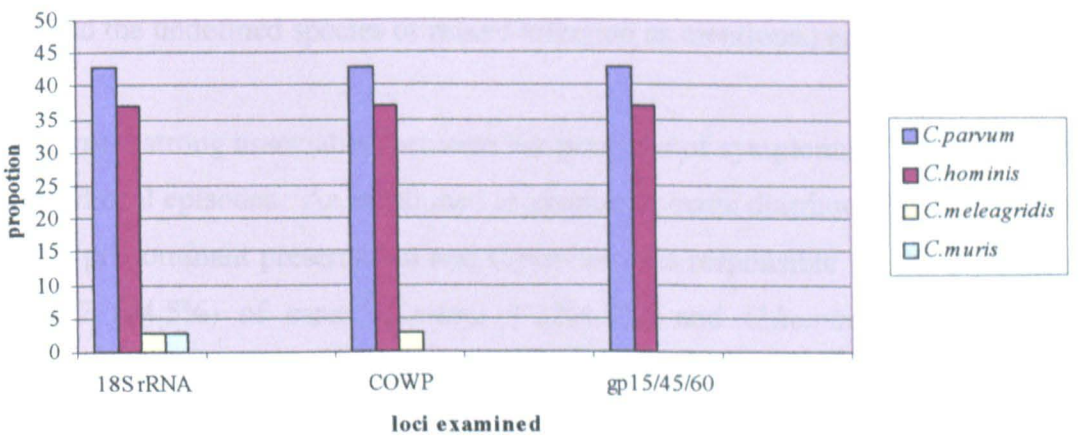
4.3.4. Comparisons of *Cryptosporidium* spp identified by the different loci examined

ELISA test was more sensitive than staining methods and PCR technique in detecting all the positive samples. PCR-RFLP for the 18S rRNA gene successfully identified all *C.parvum* isolates 15/35 (42.9%), *C.hominis* 13/35(37%), *C.meleagridis* 1/35(2.9%) and *C.muris* 1/35(2.9%). For the COWP gene target, the primers used amplified the DNA from all *C.parvum* ,*C.hominis* and *C.meleagridis* isolates. However, they failed to amplify DNA from the *C.muris* isolate, which was identified, previously by amplifying the fragment of the 18S rRNA gene for this species. All the *C.parvum* and *C.hominis* isolates were amplified by using the primers of gp/15/45/60 gene. These fragments subsequently will be sub-genotype after sequencing analysis

Table 4.10. Comparison between the different diagnostic methods.

	ELISA	ZN&AP	PCR based on the 18S rRNA gene
Cases (n=230)	P=22 N=208	P=15 N=215	P=20 N=210 (*C.parvum*, *C.hominis*, *C.muris* and *C.meleagridis*)
Controls (n=322)	P=2 N=320	P=0 N=322	P=2 N=320 (*C.hominis*)

Fig 4.7. *Cryptosporidium* species identified in the study population



4.3.5. The relationship of *Cryptosporidium* species to some of the independent variable studied

A total of 5 /24 (20.8%) *Cryptosporidium* positive samples identified in this study were collected in the winter during the months of October–January 2001. From these samples we detected 3/5(60%) *C.hominis* and 2/5(40%) *C.parvum* by the multilocus analysis used.

In the spring time (during the months of February-April 2002), 19/24 (79.2%) samples had been collected and there were a high proportion of *C.parvum* infections 10/19 (52.6%). However, *C.hominis* infections were detected in 3/19(15.8%), *C.meleagridis* in 1/19(5.3%) and *C.muris* in 1/19(5.3%). Four samples did not yield any PCR product (Table 4.10).

Table 4.11. Infection distribution in two seasons

Infection	Winter n=5	Spring n=19	χ^2	OR	95% CI	*P*
C.hominis	3(60)	3(15.8)	2.8	0.17	0.01-2.1	0.09
Zoonotic	2(40)	12(63.2)	5.72	6	0.46-101	**0.01**

In a second visit to the area between December 2002 and January 2003, a total of 11 more *Cryptosporidium* positive samples were collected; *C.parvum* 3/11 (27.3%) and *C.hominis* 7/11(63.6%) were the only species identified, and one sample exhibited the undefined species or mixed infection as mentioned earlier.

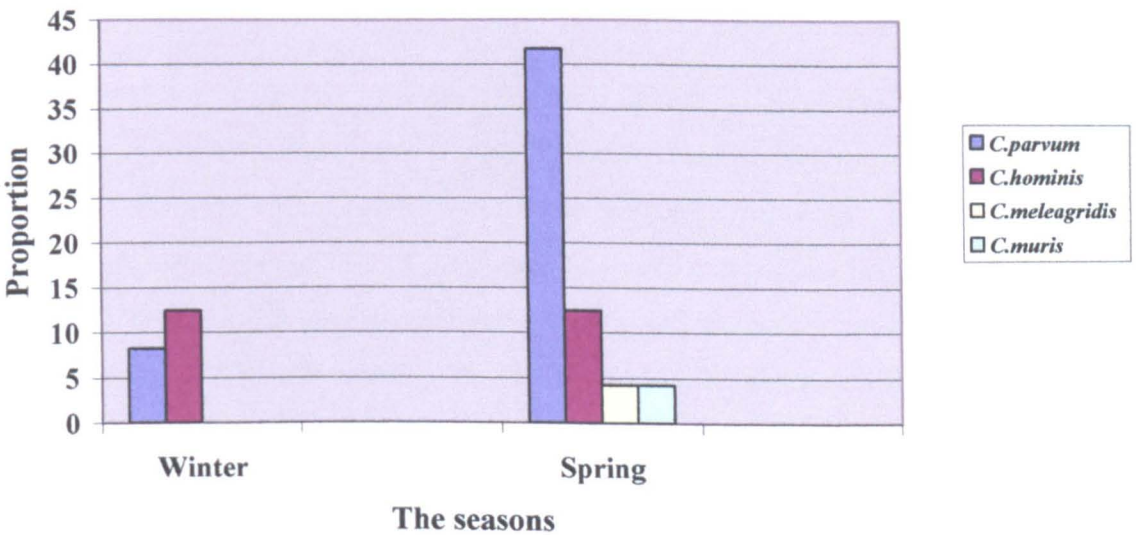
There was a strong association between the presence of symptomatic infection and the diarrhoeal episodes. As mentioned in chapter 3, acute diarrhoea 21/22 (95.5%) was the predominant presentation and *C.parvum* was responsible for the diarrhoea in 12/22 (54.5%) of cases, *C.muris* 1/22(4.5%) and *C.hominis* 4/22(18.2%). *C.meleagridis* was the cause of the case of persistent diarrhoea found in this study. *C.hominis* was detected in the two sub-clinical cases.

A strong association was also observed between the infection and being in contact with other people in the house suffering from diarrhoea. A total of 10/24 (41.7%) patients had been in contact with other persons with diarrhea. Patients having this contact are more likely to have *C.homini* infection. A relationship was also found between keeping domestic animals (cats, chickens and birds) and cryptosporidiosis infection. Five of the patients (20.8%) with cryptosporidiosis kept pets. Patients having pets are more likely to have the zoonotic infection, however theses relations are not statistically significant. No major differences was observed between patients using tap water and type of infection (Table 4.11)

Table 4.12. Risk factors of cryptosporidiosis in relation to the different species

Risk factors	***C.hominis* n=6**	**Zoonotic n=14**	χ^2	**OR**	**95% CI**	***P***
Contacting other diarrhoea	4(66.7)	6(42.9)	0.9	2.67	0.26-31.8	0.3
Contacting animals	1(16.7)	4(28.6)	0.32	0.50	0.02-7.86	0.5
Using tap water	3(50)	7(50)	0.00	1.00	0.10-9.8	0.6

Fig 4.8. Seasonal distribution of the identified species



These results had demonstrated that the predominant *Cryptosporidium* spp in the Jeddah city are *C.hominis* and bovine *C.parvum* with accounting for 28/35(80%) of the cases of cryptosporidiosis.

The most frequently detected *Cryptosporidium* spp was *C.parvum* (bovine genotype) which was found in 15/35 (42.9%) of cases of cryptosporidiosis. *C.hominis* infection was found in 13/35 (37%) samples. Other zoonotic species of *Cryptosporidium*, *C.meleagridis* and *C.muris* were identified in 1/35 (2.9 %) each of cases of cryptosporidiosis.

4.4 DISCUSSION

The faecal samples were all freshly collected and rapidly frozen at –80°C without any preservatives and by using a commercial kit (QIAMP) a high rate of *Cryptosporidium* amplification was obtained in a simple and reproducible fashion. This confirms previous observation of Bialek and Others (2002).

Of the PCR negative samples only 4 out of the 35 samples positive for *Cryptosporidium* spp failed to produce amplicons. One sample was positive by ZN, AP staining and by ELISA techniques, the other three samples were positive only by ELISA alone. The failure of these samples in not yielding any products was probably the result of a number of factors: the relatively low oocysts count in some of the samples; the presence of excess inhibitors since faecal constituents such as bilirubin, bile salts and complex polysaccharides inhibit PCR even when they are present at low concentration (Widjojoatmodjo *et al.*, 1992; Monteiro *et al.*, 1997); incomplete lysis of the oocysts; degradation of the DNA during storage and the extraction of no *Cryptosporidium* DNA or of an insufficient amount. Alternatively three of the samples could be ELISA false positive results. However, this was unlikely to occur as the efficiency of ELISA in detecting all the microscopy positive samples was very high. The oocysts observed in the microscopy positive sample could be empty oocysts. However, the insufficient amounts or/and degradation of the DNA could strongly attribute to the failure of this sample. Moreover, variation in the primer target region as may occur in novel species may result in lower specificity and amplification failure.

PCR –RFLP analysis of the 18S rRNA gene has proved to be a useful tool for differentiating *Cryptosporidium* isolates, variations in the gene being adequate for the identification of the species and sub-species of the parasite (Xiao *et al.*, 1999c). By using similar analysis of this section of the gene, it has been possible to differentiate the various *Cryptosporidium* species and genotypes reliably and to identify novel species in humans (Xiao *et al.*, 1999c; Morgan *et al.*, 1999a; Guyot *et al.*, 2001; Gatei *et al.*, 2002a; Ong *et al.*, 2002). This technique may also require further standardisation or optimisation for specific species or genotypes, as stated previously by Widmer and

Colleagues (1998c), that the sequence polymorphisms in the18S rRNA locus could cause a failure in DNA amplification. However, the primers used for this gene are based on highly conserved regions (Xiao *et al.*, 1999c) of the genus *Cryptosporidium* and they are widely applicable. The nested PCR protocol used in this study for the 18S rRNA gene also offers increased confidence in confirmation of the identity of the PCR product by successful annealing of the internal nested set of primers. Further confidence in the assay is increased by use of two restriction endonucleases, since cleavage of the PCR product is only possible at specific predicted recognition sequence sites.

With the use of RFLP a reliable differentiation different *Cryptosporidium* species and genotypes was achieved. This target gene has the advantage 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 TRAP– C1 and ITS genes, which target genes that are specific for each species and they require different primers for various species and genotypes of *Cryptosporidium* (Xiao *et al.*, 1999c).

The use of the COWP gene for PCR analysis might be less reliable than the use of the 18S rRNA as published results indicated that the 18S rRNA gene is biologically more highly expressed than the COWP gene (Spano & Crisanti, 2000a).There are five copies per haploid genome for 18S rRNA and thus the possibility of obtaining a PCR reaction with this gene (Kato *et al.*, 2003) is greatly increased.

In contrast, in the case of the COWP gene, there is only a single copy per genome. However, the amount of DNA used for amplifying the COWP locus was significantly increased from 2µl (for the 18S rRNA locus) to 10µl and this increased the sensitivity for obtaining amplicons for the samples. In addition, unlike most other genotyping tools that are based on sequences of antigen genes, the COWP technique was shown to have the ability to amplify and detect *Cryptosporidium* parasites other than *C.parvum*; thus it has been suggested that this technique may have potential in the differentiation of a broader range of *Cryptosporidium* spp. For instance, Chalmers and Others (2002a)

found a novel genotype in sheep that was microscopically indistinguishable from strains associated with human infection. However, we failed to amplify it from *C.muris*. The fact that there was no amplification in *C.muris* is to be expected and may be judged by the extent to which the COWP sequence in *C.muris* diverges from *C. parvum* upon which the primer sequences were designed. Xiao and colleagues (2000b) observed that the sequence from *C.parvum*-related parasites exhibited sequence polymorphism specifically in the reverse primer region and it was the likely cause of the failure of the DNA PCR amplification from the divergent *C.muris*.

Presently neither of the primer pairs investigated has the ability to detect efficiently all human-pathogenic *Cryptosporidium* parasites in clinical samples. Modifications will be needed for the diagnostic COWP primers to effectively detect these divergent *Cryptosporidium* parasites in clinical samples. This was probably because there is a random distribution of mutations across the entire COWP gene such that distantly related *Cryptosporidium* parasites cannot be detected (Xiao *et al.*, 2000b). Presently, the utility of the COWP–based PCR-RFLP technique in the analysis of environmental samples is probably limited because of the narrow spectrum of *Cryptosporidium* parasites detectable. However, if genus-specific primers are found, the COWP gene would be a good target for species differentiation and genotyping of *Cryptosporidium* parasites.

In this study the highly polymorphic single-copy gene, encoding gp 15/45/60 was amplified from each of the *C.parvum* and *C.hominis* isolates. This gene encodes a precursor protein (as mentioned earlier) that is proteolytically cleaved to yield mature cell surface glycoproteins gp 45 (also named gp 40) and gp 15, both of which are implicated in zoite attachment to and invasion of enterocytes. The nucleic acids sequence analysis of this locus from 29 geographically diverse *C.parvum* and *C.hominis* has been studied previously by Strong and Others (2000) who showed that it was highly polymorphic, much more so than any *Cryptosporidium* locus examined to date. The locus manifested numerous single nucleotide and single amino acid polymorphisms (SNPS and SAAPS) particularly among *C.hominis* isolates.

The polymorphisms observed at this locus are not generated through the use of multiple gene copies as confirmed earlier (O'Connor *et al.*, 2002). However these findings are not surprising in light of the biology of *Cryptosporidium* infection. Some apicomplexans, such as *Plasmodium falciparum* are capable of establishing persistent infection through rapid variation of surface antigens (Saul, 1999). The genes encoding these antigens are usually present at a very high copy number (Su *et al.*, 1995).

Previous investigations have examined genetic diversity at many *Cryptosporidium* loci, including the 18S rRNA (Xiao *et al.*, 2001), the ITS1 region of rRNA (Morgan *et al* ., 2000a), and the genes encoding HSP70(Sulaiman *et al.*, 2000), acetyl coenzyme A synthetase (Morgan *et al.*, 1998b), COWP (Spano *et al.*, 1997b), and TRAPC1 and C2 (Spano *et al.*, 1998; Elwin *et al.*, 2001). In each case these studies have identified two predominant species, one exclusively associated with isolates derived from human infection (*C.hominis*) and one associated with isolates derived from both human and animal infections (*C.parvum*).

In this study we found that the majority of infections were caused by *C.parvum* 15/35 (42.9%) as defined by the PCR-RFLP analysis at the 18S rRNA and the COWP loci and will be confirmed by the sequence analysis at the gp15/45/60 locus. The presence of fifteen *C.parvum* strains is significant and, moreover, this is not in agreement with the epidemiologic studies in which *C.hominis* isolates have been shown to be more frequently identified in immunocompetent as well as HIV-infected humans (Widmer *et al.*, 1998c). However, other studies conducted in the UK, France and the Netherlands have shown that *C.parvum* was the predominant species (Patel *et al.*, 1998; Quiroz *et al.*, 2000; Guyot *et al.*, 2001Glaberman *et al.*, 2002 Gatei *et al.*, 2003).

C.parvum has not only been detected in humans but also in a wide range of livestock and wild animals, whereas *C.hominis* appears to be restricted largely to humans, although there are published reports of natural infection in a non-human primate and a dugong (Morgan *et al.*, 2000b) and experimental infections in pigs (Widmer *et al.*, 2000), lambs (Giles *et al.*, 2001) and calves (Akiyosh *et al.*, 2002). Despite this,

C.hominis has a far more restricted host range than *C.parvum* and the detection of *C.hominis* in a sample indicates a high probability of a human source (Patel *et al.*, 1998)

C.meleagridis normally infects turkeys. However, the species has also been recently confirmed in an Indian ring-necked parrot, a common aviary bird with a worldwide distribution (Morgan *et al.*, 2000c). *C.meleagridis* may therefore have a much wider host range than was previously considered possible.

This study represents the fourth confirmed case of *C.muris* infection in humans with acute diarrhoea. Previously, one case of *C.muris* infection was identified in an HIV-positive child in Thailand (Tiangtip & Jongwutiwes, 2002), an HIV-positive adult in Kenya (Gatei *et al.*, 2002a) and in an HIV-infected woman in Peru (Palmer *et al.*, 2003) using microscopy and molecular analysis, *C.muris* was also found in two healthy Indonesian girls, but the diagnosis was not confirmed by molecular tools (Katsumata *et al.*, 2000).

In the present study, there were associations between animal ownership among families whose children had pets versus species of human *Cryptosporidium*. Five subjects in the study population had a pets, from these 80 % had a zoonotic cryptosporidiosis infection. In any case we cannot say with certainty that the so-called zoonotic types of *Cryptosporidium* were transmitted directly from animals to child, rather than via contamination of water, food or hands with animal or human faeces. Previous reports that identified *C.parvum*, *C.canis*, *C.felis* and *C.meleagridis* indicated that children reporting contact with animals were almost all infected by the zoonotic species (McLauchlin *et al.*, 2000; Pedraza-Diaz *et al.*, 2001; Xiao *et al.*, 2001). Indeed, direct transmission of *C.parvum* from animals to human is well documented (Miron *et al.*, 1991; Millard *et al.*, 1994; Peng *et al.*, 1997). Moreover, 66.7% of *C.hominis* cryptosporidiosis infection was in patients who had a contact with other person in the households having diarrhoea and this could in part explain the role of person to person transmission.

It is not clear whether the extensive zoonotic origin of *Cryptosporidium* parasites infecting humans is a typical occurrence in the Jeddah area as there are no other reports from Saudi Arabia identifying species and genotypes of *Cryptosporidium* infection. There are some reports, however, which are based on a hospital case and community studies that identified the occurrence of *Cryptosporidium* oocysts only as a pathogen (Khan *et al.*, 1988; Bolbol, 1992; Al-Braiken *et al.*, 2003). However, we believe from the results of this study that direct contact with the animals can increase the prevalence of the zoonotic infection.

The number of zoonotic isolates, including *C.parvum* bovine genotype, is potentially high especially in that the samples were from non- HIV infected persons. However, Chalmers and Others (2002b) indicated that the infection with unusual types of *Cryptosporidium* is not restricted to immunocompromised patients. The zoonotic isolates - excluding *C.parvum* bovine genotype - recovered from humans, world wide, remain few (Fayer *et al.*, 2000). However, Gatei and Others (2002b) found that zoonotic species were as common as *C.hominis* in HIV-infected Thai persons. Moreover, a higher prevalence of *C.meleagridis* than bovine genotype has also been observed in immunocompetent children in Peru (Xiao *et al.*, 2001).

The application of multilocus gene analysis should help determine the level of intraspecific variation in the genus *Cryptosporidium*. However, sequence analysis would be crucial in confirming the identity of the isolates and to classify the different alleic subgroups of *C.hominis* based on gp15/45/60 gene.

CHAPTER FIVE

NUCLEOTIDES SEQUENCING AND PHYLOGENY

5.1. INTRODUCTION

Sequencing and phylogenetic analysis of DNA or protein sequences has become an important tool for studying the evolutionary history of and the relationship between a number of organisms from viruses to humans (Nei & Kumar, 2000). Phylogenetic analyses of various isolates of *Cryptosporidium* at a number of different loci has provided strong evidence that this genus is composed of several distinct and valid species (Morgan *et al.*, 1999a). Previous studies of the 18S rRNA gene have shown that the locus can be useful for specific identification of most *Cryptosporidium* species (Widmer *et al.*, 1998c; Morgan *et al.*, 2000a; Xiao *et al.*, 2001). There has also been extensive application of analysis of this gene in the phylogeny of *Cryptosporidium* with indications that the presence of hetrogenous copies within the genome of a single organism does not affect the overall phylogentic position of the organism (Morgan *et al.*, 1997; Xiao *et al.*, 1999b). Previous studies indicated that various *Cryptosporidium* spp and host adapted *C.parvum* strains have extensive sequence polymorphisms in the COWP gene which seem to reflect the genetic relatedness of different *Cryptosporidium* parasites. These results provide useful information for COWP-based molecular differentiation of *Cryptosporidium* spp and genotypes (Spano *et al.*, 1997b; Xiao *et al.*, 2000b). A remarkable degree of genetic heterogenetity at the gp15/45/60 locus has been demonstrated among *C.hominis* isolates in a cohort study of children in South Africa by Leav and Others (2002) and, moreover, this study confirmed the presence of extensive nucleic acid and amino acid sequence polymorphisms. This study (Leav *et al.*, 2002) initially identified isolates by amplifying the 18S rRNA and COWP target genes. Recently Alves and Others (2003) described seven alleles based on extensive sequence polymorphisms in the gp15/45/60 gene and within each allele, there are different subgenotypes based on the number of a trinucleotide repeat.

The aims of this chapter are to confirm the identity of the isolates identified in the present study and to assess the genetic diversity and Phylogeny of *Cryptosporidium* isolates from Saudi Arabia, sequence analysis was carried out based on different genetic loci (18Sr RNA, COWP and gp15/45/60 genes).

5.2. MATERIAL AND METHODS

5.2.1. Sequencing

Representative PCR amplicons (Table 5.1) were chosen from each of the species and genetic loci. These samples were first purified by eluting the PCR product from a low melting point agarose gel. A 1.2% low melting point agarose gel (Gibco BRL, Life Technologies, Scotland) was prepared and 50µl of the PCR products (secondary PCR product for18S rRNA) were loaded in the wells. These PCR products were electrophoresed at 120 volts and visualized by ultraviolet trans-illumination having stained them with ethidium bromide as described previously in Chapter 4.

5.2.2. Purification of the PCR products

A commercial purification kit (Sigma Gen Elute Mini- prep (Sigma chemicals, UK) was used for DNA purification from the low melting point agarose gel. According to the manufacturer's instructions, the DNA fragments (833-837 bp for 18S rRNA, 550 for COWP and 1kb for gp15/45/60) were excised from the low melting point agarose gel by using a clean sharp scalpel; they were put into eppendorf tubes and weighed. To dissolve the gel 3 volumes of gel solubilizer (Guanidine thiocyanate, provided in the kit) were added to 1 volume of the gel slice and incubated at 50°C for 10 minutes. To bind the DNA, the samples were loaded into the Gene Elute Mini prep binding column and centrifuged for 1 minute at 1300 xg, to allow the dissolved agarose together with the primers flow through the column. The DNA was washed by adding 600µl of washing solution to the binding column. Finally the DNA was eluted from the column by using 50-70µl of HLPC water.

The recovered DNA samples were then directly sequenced using two directional primers (reverse and forward) for increased sequence accuracy by using an automated DNA sequencer (ABI Automatic Sequencer, Lark Technologies, UK) after confirmation of the presence of the DNA by running 8µl from each sample on an agarose gel.

Table 5.1. The representative samples sequenced from each gene group.

Species	18S rRNA	COWP	gp/15/45/60
C.parvum	6	6	5
C.hominis	4	3	6
C.meleagridis	1	1	-
C.muris	1	-	-

Table 5.2. The sequences used for comparisons from the GenBank.

Accession No	Strain	Species	Locus
AF108864	C1	*C.parvum* bovine	18Sr RNA
AF108865	H7	*C.hominis*	18S rRNA
AF093497	CMU03	*C.muris*	180S rRNA
AF404821	1021A	*C.meleagridis*	180S rRNA
AF248742	human	*C.meleagridis*	COWP
AF266265	181	*C.hominis*	COWP
AF266273	3	*C.parvum* bovine	COWP
AF022929	NiNc-1	*C.parvum* bovine (IIa)	gp 15/45/60
AF440624	4	*C.hominis* (Id)	gp 15/45/60
AF164498	610542J	*C.hominis* (Ib)	gp 15/45/60
		C.parvum (Ic)	gp 15/45/60
AF440621	1		
AF440634	16	*C.hominis* (Ia)	gp 15/45/60

5.2.3. Sequence analysis

The chromas programme version 1.45 (1996; C.MacCarthy Griffith University, Southport, Queensland Australia) was used to read all the amplicon sequences. DNASTAR version 4.05 (1993-2000) was used for editing the consensus sequences. To confirm the identity of the sequences from the GenBank, Blast searches (WU-2 EMBL) were undertaken. Multiple alignments of the DNA sequences were done with the CLUSTALX program (EMBL, Heidelberg, Germany). Two types of phylogenetic analyses were used on the aligned sequences. The DNA distance based Neighbor Joining (NJ) and Maximum Likelihood (ML) analysis by using the phylogenetic analysis software (PHYLIP version 3.5c) (© J.Felsenstein and the University of Washington). Tree reliability was assessed by the bootstrap method with 2000 replicates by using the software Mega-2 (Kumar *et al.*, 1993). Dna-SP version 3 (Rozas & Rozas, 1999) was used to calculate the nucleotide diversity between different isolates at the 18S rRNA and COWP gene loci.

5.2.4. Cloning

The PCR amplicons of 18S rRNA (secondary PCR), COWP, and gp15/45/60 fragment genes from isolate C 115 were cloned into pGEM-T, to determine whether this isolate was from a novel species or mixed infection with different *Cryptosporidium* spp. This procedure involves ligation of the DNA fragment into a plasmid vector encoding markers such as enzyme activation (β-galactosidase) or antibiotic resistance (β-lactamase). In this case when the ligation is successful when grown on appropriate agar plates bacterial colonies are white while the rest of the colonies are (non-transformer) blue. To yield a more complete picture 60 transformed colonies (20 from each gene)were selected at random and grown to larger numbers in Luria broth.

Procedure

A commercial pGEM-T Easy cloning kit (Promega, Madison, USA) was used. All the reagents and the instructions were provided. The test DNA was cloned into the pGEM-T plasmid vector and transformed into competent *Escherichia coli* TG2 cells.

5.2.4.1. Preparation of competent *E.coli* TG2 cells

Reagents

RF1 Buffer

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

RF2 Buffer

10mM MOPS	(Sigma Adrich, UK)
75 mM $CaCl_2$	
10mM RbCl	
15% glycerol	

Procedure

E-coli TG2 (Transformation system bacteria) were made transformation competent by incubating them with RF1 buffer on ice for 15 minutes followed by centrifugation at 1300 xg for 5 min after decanting the supernatant, the obtained pellet was incubated on ice with buffer RF2 for 30 mins. The competent bacteria were then dispended into aliquots (200µl each) and stored at −20°C until used.

5.2.4.2. Cloning of PCR product into pGEM-T

Approximately 100ng (3µl) of purified PCR product (for each gene locus) was incubated with 2x rapid ligation buffer, pGEM-T vector (50ng) and T4 DNA ligase (all provided in the kit) in a total volume of 10 µl for 1 hour on the bench. Methods were as described in the Promega manual (Promega, pGEM-T Vector System I).

5.2.4.3. Transformation

The pGEM-T vector with the insert DNA was transformed into competent TG2 cells by incubation on ice (1hour), heat shocking (42°C for 45 sec), incubation on ice (5 min) and growth at 37°C in Luria broth (LB-10g Bacto-tryptone-5g Bacto-yeast extract, 5g NaCl (1 Litre)) for 45 minutes. Then the bacteria were plated onto LB-agar plates with 100µg/ml Ampillicin (Sigma Adrich, UK) 80 µg/ml X-Gal (5-bromo-4-chloro-3 indolyl-β-D galactopyranoside) and 200µg/ml IPTG (isopropyl-β-D thiogalactopyranoside, (Sigma Adrich, UK) and grown at 37°C overnight.

The screening for the presence of the functional β-galactosidase gene or its absence involves the addition of a lactose analogue called X-Gal plus an inducer of the enzyme (IPTG), the former is broken down by β-galactosidase to a product that is colored deep blue. Non-recombinant colonies, the cells of which synthesize β-galactosidase will be colored blue whereas recombinants will have a disrupted gene and be unable to make β-galactosidase. These colonies will be white. Only bacteria containing the vector with the PCR product inserted will grow as white colonies.

Colonies containing the vector with the target cloned PCR product were grown in 500mls of Luria broth overnight at 37°C. A total of 60 transformed colonies for all targets (20 for the 18S rRNA, 20 for the COWP and 20 for the gp15/45/60) were then screened by PCR with the primers previously mentioned in chapter 4 (AL 1598 and AL 3032 for 18S rRNA, Cry15 and cry 9 for COWP and gp15 ATG and gp 15 STOP for the gp15/45/60 genes).

5.2.4.4. Recovery of the DNA from transformed bacteria

By using the QIA Prep spin Miniprep kit (QIAGEN, UK) DNA was recovered from the transformed bacteria according to the manufacturer's instructions. The test DNA was then recovered by using a spin column membrane and a high salt buffer (all provided) that bind the DNA into the membrane. The DNA is then eluted from the membrane by using 50 µl HPLC water. Approximately 1 µg of the test DNA for each target was dispensed into an eppendorf tube and sequenced with vector primers

M13-20 GTAAAACGACGCCCAGTGAG
and M13-rev GGAAACAGCTATGACCATG.

5.2.5 Nucleotide diversity and phylogeny

Analyses of evolutionary distances are fundamental tools for the study of molecular evolution and are useful for phylogenetic reconstruction. Genetic diversity refers to the variation at the level of individual gene (polymorphism). It is often said that variation in genes is necessary to allow organisms to adapt to ever changing environments. It is actually the variation in alleles that is critical; new alleles appear in a population by the random and natural process of mutation and the frequency of occurrence of an allele changes regularly as a result of mutation, genetic drift and selection (Futuyma, 1998). The polymorphism is usually measured by the probability that alleles chosen from the population will be different.

To analyze genetic polymorphisms within the Saudi *Cryptosporidium* isolates based on the different gene loci, the Tajima Neutrality test was carried out (Tajima, 1989). This test is based on the neutral theory of molecular evolution which predicts that regions of the genome that evolve at high rates will also present high levels of polymorphism within species (Nei & Kumar, 2000).

The relative distances between different *Cryptosporidium* spp and genotypes were calculated by using the Kimura – 2 parameter method. In this method the number of nucleotide or amino acid substitutions between pairs of sequences are measured.

Phylogenetic relationships of genes or organisms are usually presented in a tree-like form either with a root (rooted tree) or without any root (un-rooted tree). These trees are used to classify and understand the diversity of organisms and to track the transmission and spread of pathogens between individuals and through populations. Trees consist of nodes and branches that connect them. The order and the length of branches may be significant. Nodes are either external at the end of branch or internal where two branches fork. External nodes represent whatever is being compared in the tree. Internal nodes represent the ancestors of the species or genes at the external nodes (Nei & Kumar, 2000). There are many statistical methods that can be used for reconstructing phylogenetic trees from molecular data. Commonly used methods are classified into three major groups: distance matrix methods, the parsimony method and the maximum likelihood method.

In distance matrix methods evolutionary distances are computed for all pairs of taxa based on the number of nucleotide or amino acid substitutions between sequences and a phylogenetic tree is constructed by using an algorithm based on the functional relationships among these distance values. Moreover, the branch lengths represent the genetic distance between any two isolates. There are many different methods of constructing trees from distance data. In the present study we used the NJ method that is based on the minimum evolution principle. This method finds the nearest 'neighbour' through nucleotide homology that when joined, minimized the total length of the tree.

The tree produced by MP analysis is that which is compatible with the smallest number of evolutionary changes between the sequences in the alignments, so it is better suited to analyse closely related sequences.

The ML method is considered by many to be the method of choice for tree reconstruction. In this method, the probability of occurrence of the patterns of alignment of nucleotide or amino acid residue substitutions are calculated (Futuyma, 1998).

The statistical significance of phylogenetic trees is assessed by Boot-strapping values. These values give the proportion of such trees that have the relevant branch point. This is done by creating new data sets by randomly choosing columns from the test alignment and combining them to form new set. A high boot strap values give some confidence on the reality of the branch point.

NJ and ML rooted trees were constructed for the amplicons from the *Cryptosporidium* isolates identified in this study to assess the relationship among various species based on the different loci examined. The reliabilities of these trees were assessed by the Boot-strap method with 2000 pseudoreplicates. A 95% cut-off was used as the statistically significant value. However, values greater than 50% are reported since the bootstrap method may give a very conservative estimate for the reliability of assignment to a clade.

5.3.RESULTS

5.3.1.Cloning

Each transformation experiment was successful in that when plated out, *E.coli* TG2 transformed with pGEM-T containing various PCR amplicons produced a mixture of blue and white colonies. The white colonies contain pGEM-T with the disrupted β-galactosidase gene were picked.

Three different PCR amplifications from the18S rRNA, COWP and gp15/45/60 loci were examined from approximately 60 different white colonies (20 for each target). Extractions that yielded products of between 833- 837 bp or 550 bp were digested by *SspI, VspI* and *RsaI* for the 18S rRNA and COWP loci respectively. For the 18S rRNA gene, 2 colonies exhibited *VspI* restriction sites with fragments of 561 and 104 bp which demonstrated the presence of *C.hominis* and 4 had fragments of 628 and 104 bp which is characteristic of *C.parvum* bovine genotype while the remainder of the colonies did not yield any PCR products. For the COWP gene, 5 colonies had the restriction sites characteristic of *C.hominis* (284,129, and 106 bp), and 5 had that of *C.parvum* bovine genotype (413 and 106 bp); 5 did not yield any products. Only 3 colonies yielded the 1Kb fragment for the gp15/45/60 gene.

Representative colonies for the 18S rRNA and COWP gene loci, and all the yielded colonies for the gp/15/45/60 gene were selected for purification and sequence analysis.

5.3.2.Sequencing identity

5.3.2.1.The 18S rRNA gene

Partial sequences from the 18S rRNA gene were obtained from *C.hominis* (4 samples) and *C.parvum* bovine genotypes (6 samples), one from *C.muris* and one from *C.meleagridis*. The lengths of the gene fragments varied from 831-837 depending on the species and genotype. The gene fragment of the *C.hominis* isolates was the longest and that of the *C.parvum* bovine genotype (gene B) was the shortest.

The 18S rRNA genes of *Cryptosporidia* were AT rich with A and T % contents of 33.2% to 30.8% within each species; however the A and T content of different isolates were quite similar to each other.

The Blast searches (WUBlast-EMBL) confirmed the finding by PCR and RFLP analyses that all isolates sequences from *Cryptosporidium* spp of differing species and genotypes. The identities of *C.parvum* bovine and *C.hominis* sequences based on 18S rRNA were 99.8-100% compared to the published strain C1 (Accession number AF108864) and 99.5-99.7% to the published strain H7 (Accession number AF108865) in GenBank respectively. *C.meleagridis* showed 100% identity to the published isolate (Accession number AF 404821). *C.muris* showed 99% sequence identity with *C.muris* isolate from a mouse strain MU03 (Accession number AF 093497) based on the 18S rRNA gene.

C.hominis/C.parvum bovine genotype isolated from the C 115 sample showed 99% sequence identities to the published strain H7 (Accession number AF 108865) and strain 1 (Accession number L 25642) respectively.

5.3.2.2. The COWP gene

Partial sequences of the COWP gene (550bp) were also obtained for *C.hominis* (3 samples), *C.parvum* bovine genotype (6 samples) and *C.meleagridis* (1 sample).

The COWP gene sequence had identities between (99.6-100 %) to the published strain 6 (Accession number AF 266273) for *C.parvum* and 99.8-100% to the published strain 181 (Accession number AF266265) for *C.hominis*. *C.meleagridis* showed 100% identity to the published isolate (Accession number AF 24872) based on this gene fragment.

Sequences for *C.parvum* and *C.hominis* obtained from C115 were 99% and 100% identical to the published strains 3 and 181 (Accession numbers AF 266273 and AF 266265) respectively.

5.3.2.3.The gp 15/45/60

Sequences of gp/15/45/60 gene (1kb) was also achieved for *C.hominis* (6 samples) and *C.parvum* (5 samples). One of the interesting aspects of the gp 15/45/60 gene is that *C.hominis* isolates each carry a polymorphic variant of the gene that is found in *C.parvum* isolates. All *C.parvum* and *C.hominis* could be divided into five alleles. Three alleles corresponded to *C.hominis* (Ia, Ib, and Id) and two alleles corresponded to *C.parvum* (IIa and Ic). These allelic groups showed (98-99%) identity to the published genotypes from isolates (16, 610542J, and 4), Accession numbers (AF 440634, AF164498, and AF440624) in GenBank. The gp 15/45/60 amplicons of all *C.parvum* bovine genotype alleles IIa and Ic showed (99%) identities to the published isolate NiNc-1 and 1 in GenBank (Accession numbers AF 022929 and AF 440621). All of these alleles are corresponded to the previously described allelic families Ia, Ib, Id, Ie, and If in *C.hominis* and IIa, IIb, Ic, and IId in *C.parvum* (Alves *et al.*, 2003). Two of the *C.hominis* (CH 68 and CH 4) isolates exhibited *C.parvum* allelic IIa genotype with 99% identities to the published strain NiNc-1 Accession number (AF 022929). To the best of our knowledge this is the first report to demonstrate such an occurrence.

Two of the gp15/45/60 amplicons from C 115 exhibited *C.hominis* Ib allele with 100 % similarity to strain 610542J (Accession number AF164498). In contrast, the other amplicon exhibited the *C.parvum* bovine genotype allele Ic with 99 % to the published sequence (Accession number AF 440621). All the isolates exhibited gp 15/45/60 alleles concordant with the 18S rRNA and COWP results. However, allele Ic that originally was described as *C.hominis* allele (Strong *et al.*, 2000) and contained the *C.parvum* bovine genotype isolated from C.115 had the *C.parvum* bovine genotype sequence in the 18S rRNA and COWP genes. This observation might raise the possibility of sexual recombination within and between *C.hominis* and the *C.parvum* bovine genotypes.

5.3.3. Phylogeny and Nucleotide diversity for 18S rRNA gene

Four different *Cryptosporidium* species have been identified in this study based on the analyses of the 18S rRNA gene. These species are *C.parvum* (bovine genotype), *C.hominis*, *C.meleagridis*, and *C.muris*. The phylogenetic relationship between these species based on analyses of 18S rRNA amplicons is illustrated in Figures 5.1 and 5.2 based on the NJ and ML phylogram methods respectively.

C.muris was used as the out-group as this species showed 87% or less similarity to the others. In the NJ method (Figure 5.1) the four *Cryptosporidium* species examined in this study formed two clades with the full statistical reliability. One clade contained *C.muris* as the out-group. The other clade consisted of *C.meleagridis*, *C.hominis* and *C.parvum* bovine genotype emerging as sister groups with bootstrap values of 81%. This phylogenetic analysis has shown that *C.muris* is the most divergent of the *Cryptosporidium* species. The *C.meleagridis* isolate was distant from the majority of the *C.parvum* bovine isolates and the *C.hominis* group with a high bootstrap value of 98%. The *C.hominis* group produced two clusters that separated from each other with full statistical reliability. All strains of *C.parvum* bovine genotypes were placed into three clusters. Gene A, gene B were each placed into a unique cluster whereas *C.parvum* isolated from C115 was placed into a separate cluster as it showed a five nucleotide substitution (bootstrap value of 97%). In the ML method, *C.meleagridis* was placed as a sister group to *C.muris* (Figure 5.2).

At approximately position 664 on the 18S rRNA gene the bovine isolates displayed the sequence TATATTTT whereas the all *C.hominis* isolates examined exhibited the recognition sequence TTTTTTTTTTT. There were three bases deletions in *C.parvum* bovine isolates CPB 129 and CPB 40 from which gene B has been amplified and which produced an 831 bp fragment instead of the 834 of gene A.

Figure 5.1. Phylogeny of *Cryptosporidium* isolates by a rooted NJ- tree based on 18S rRNA

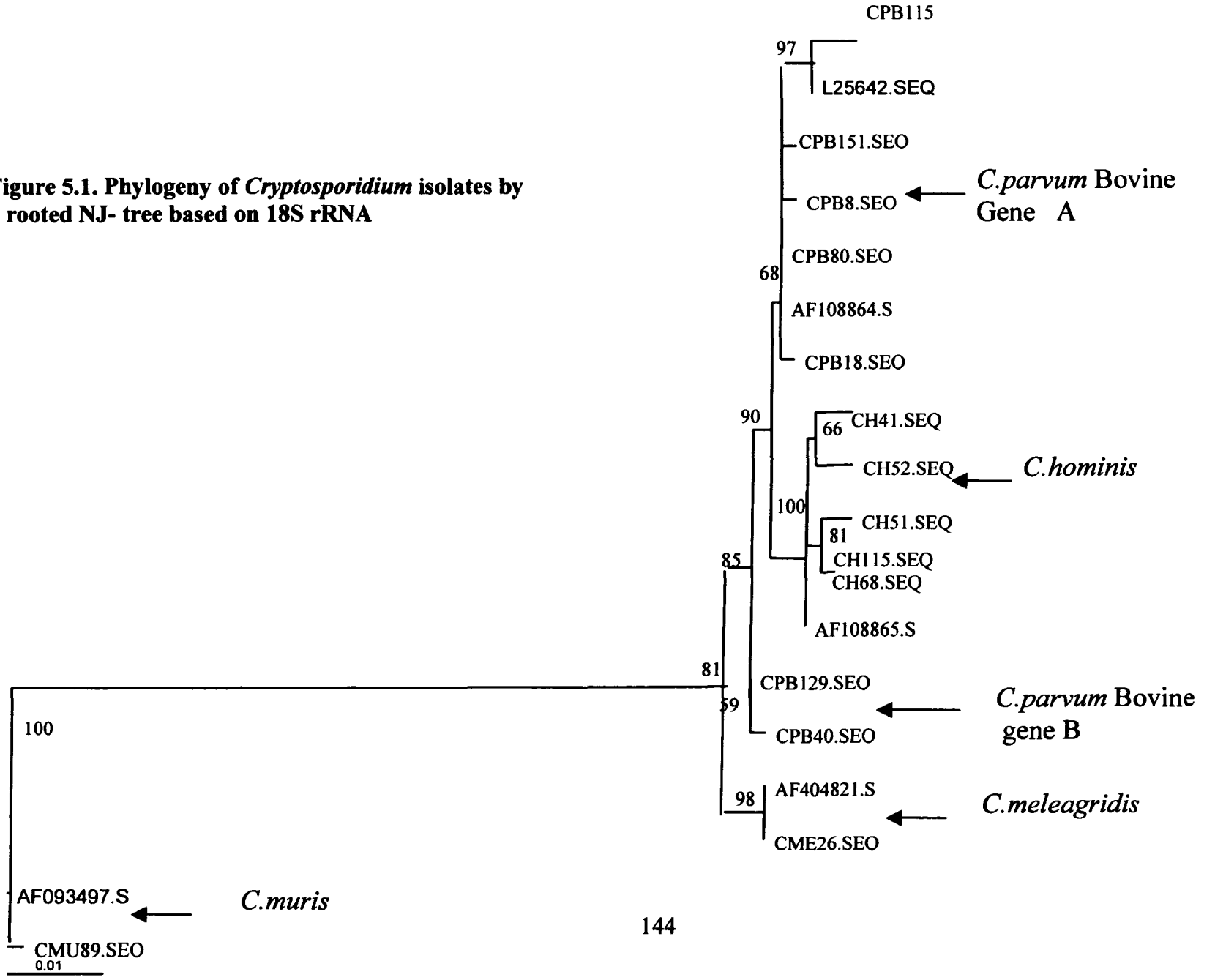


Figure 5.2.Phelogeny of *Cryptosporidium* isolates by a rooted ML-tree based on 18S rRNA

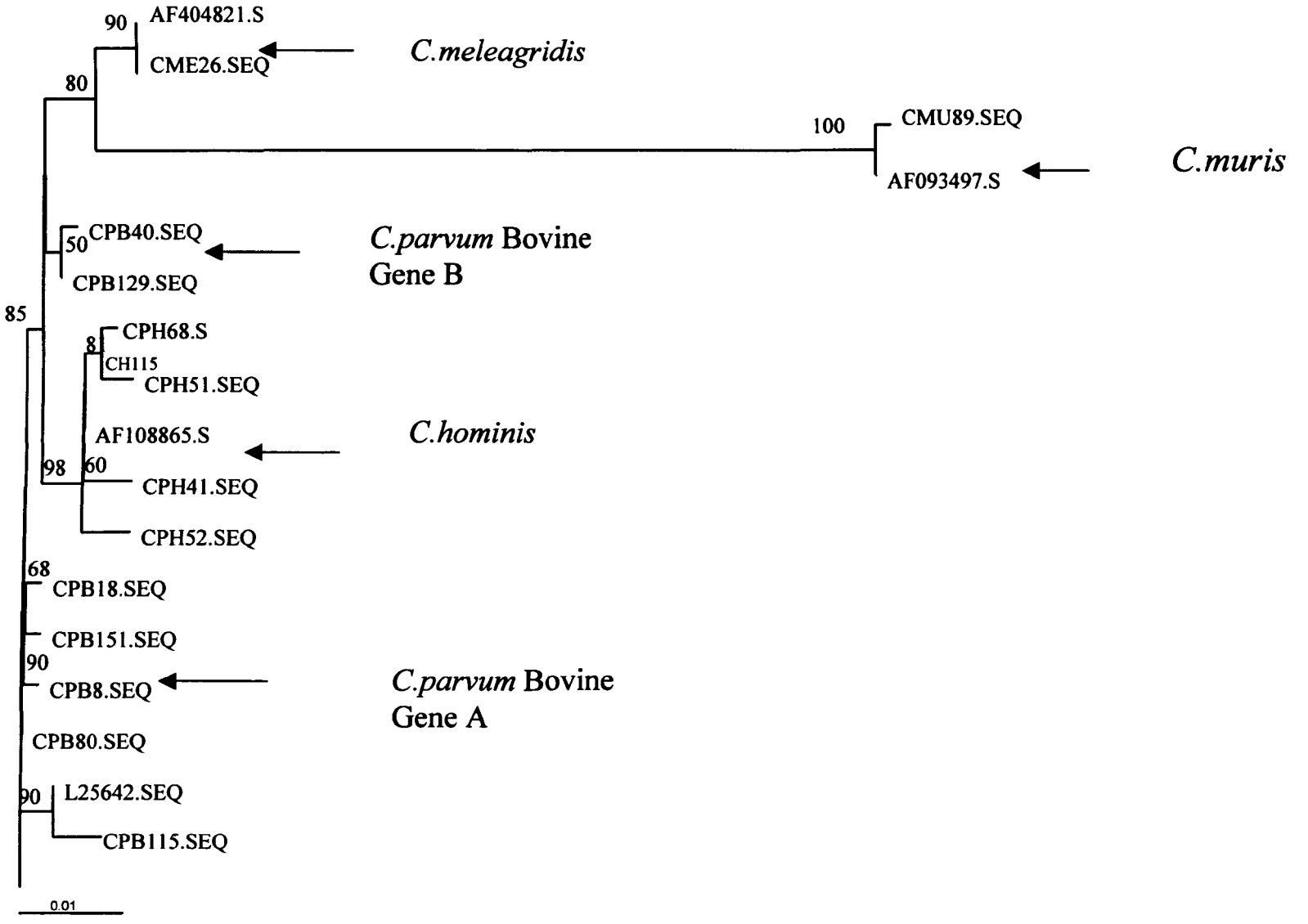


Table 5.3. Evolutionary genetic distance among selected *Cryptosporidium* isolates based on 18S rRNA CPB 80 was used as a consensensus.

Distance method: Nucleotide: Kimura 2-parameter [Pairwise distances] Standard Error estimated by bootstrap method, Replications = 500 (Mega-2).

Species	Type		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1-*C.parvum*	Bovine	CPB80	-													
2-*C.parvum*	Bovine	CPPB151	0.2													
3-*C.parvum*	Bovine	CPB115	0.8	1.0												
4-*C.parvum*	Bovine	CPB8	0.2	0.3	1.0											
5-*C.parvum*	Bovine	CPB18	0.2	0.3	1.0	0.3										
6-*C.parvum*	Bovine	CPB129	0.3	0.5	1.1	0.5	0.5									
7-*C.parvum*	Bovine	CPB40	0.5	0.6	1.3	0.6	0.6	0.2								
8-*C.hominis*		CH41	0.9	1.1	1.8	1.1	1.1	0.9	1.1							
9-*C.hominis*		CH52	0.9	1.1	1.8	1.1	1.1	0.9	1.1	0.8						
10-*C.hominis*		CH68	0.8	0.9	1.6	0.8	0.9	0.8	0.9	0.8	0.8					
11-*C.hominis*		CH51	0.9	1.1	1.8	1.1	0.9	0.9	0.9	0.9	0.9	0.5				
12-*C.hominis*		CH115	0.9	1.1	1.3	0.9	0.9	0.8	0.8	0.9	0.9	0.5	0.9			
13-*C.meleagridis*		CME26	0.9	1.1	1.0	0.3	0.2	0.8	0.9	1.7	1.7	1.6	1.7	1.8		
14-*C.muris*		CMU89	8.0	8.2	9.0	8.2	8.2	7.7	7.9	9.1	9.1	8.9	8.9	9.1	8.0	-

Figure 5.3. Summary of variable sites for selected sequences (Number of variable sites 68) based on 18S rRNA

```
1-CP80    ATACGTGATA ATTTTTTAAA AATTATATAC TAATAATCTA ATTTTTATAT ATACATGAAT TCAAGCAG
2-CP151   ....A..... .......... .......... .......... .......... .......... ........
3-CP115   .......... .......... .......... ..C....... ......G... .......... ....CGG.
4-CP8     .......... .......G.. .......... .......... .......... .......... ........
5-CP18    .....C.... .......... .......... .......... .......... .......... ........
6-CP129   ......... .......... .......C.. .......... T......... .......... ........
7-CP40    .......... .......... .......C.. .......... T......... .......... .......C
8-CH41    ......A..C .......... .........G .........T T......... ...T...... ........
9-CH52    .C....C... .........C .......... .........T T......... ...T...... ........
10-CH68   .....A.... .......... C......... .........T T......... ...T...... ........
11-CH51   ...A...... .......... C......... .........T T......... ...T...... .T......
12-CME26  .......... .......... .......... ........A. TA........ ......A... ........
13-CMU89  C.T....GC. GCAACCG.G. .GCGGAT.T. AC.CTCCT.. T.CAGC.ACA GCG.GCAGGC C.GC....
```

The CP 80 isolate (similar 100% to the published isolate AF 108864) was used as the consensus sequence. Isolates from 2 - 7 are *C.parvum* bovine genotype, isolates from 8 -11 are *C.hominis*, isolate 12 is *C.meleagridis* and isolate 13 is *C.muris*.

Dna SP (version 3) was used to assess the diversity between the isolates based on the nucleotide substitution and the mutation rate. Usually nucleotide substitutions occur at random among the four nucleotides. The 18S rRNA gene does not code for a protein and can thus be aligned only at the nucleotide level and the average number of nucleotide substitutions per site can be calculated within each species. The results of examining diversity within each species revealed that the variation was significantly high in the *C.parvum* bovine genotype. A total of 11 mutations (Eta) and a nucleotide diversity (Pi) of 0.00558 was observed in this genotype. These results demonstrate the extensive diversity within *C.parvum* bovine genotype (Tajima D-value = −1.16366, *P*=0.10). The reason for the extensive nucleotide diversity in this genotype was demonstrated by the high number of singleton sites (9) and a high mutation rate per sequence (4.9). *C.hominis* also showed a high mutation rate of 10. These results are shown in Table 5.4.

No such results are available for *C.muris* and *C.meleagrids* as at least four different sequences are required for comparison. Figure 5.3 shows in summary the variable sites for selected sequences. These sequences were aligned in relation to CPB80 that had shown 100% identity with *C.parvum* strain C1 (Accession number AF108864).

Table 5. 4. Nucleotide diversity and mutation rates of some of *Cryptosporidium* isolates at the 18S rRNA gene fragment.

Isolates	No of Seq	Total sites	S	Eta	Eta (S)	Theta /Eta (S)	Pi	Theta /Seq	Theta /site	Tajimas D-value	*P*
C.hominis	4	643	9	10	8	6.750	0.0078	4.409	0.00763	**-0.83379**	**0.10**
C.parvum (Bovine)	7	641	11	11	9	7.714	0.00558	4.990	0.00712	**-1.16366**	**0.10**

S =Number of polymorphic sites
Pi =Nucleotide diversity
Eta =Total Number of mutation
Eta(S) Total number of segregating sites
Theta/Eta(S)= mutation rate per singleton site
Theta/Site=mutation rate per nucleotide site
Theta/Seq=mutation rate per sequence
TajimaD-value=neutrality test
P-value=level of significance differences between sequences

5.3.4. Phylogeny and nucleotide diversity for the COWP gene

The phylogenetic relationships between the COWP loci of the *Cryptosporidium* isolates was assessed in Figures 5.4 and 5.5 with the use of previously described tree construction methods. Three different species were identified based on this locus. These species are *C.parvum* (Bovine genotype), *C.hominis* and *C.meleagridis*.

As mentioned in Chapter 4, no COWP-PCR amplicon was obtained from *C.muris*.
The tree was anchored by using *C.meleagridis* as the out-group as this species showed 97% or less similarity to the other species. The other two species formed one clade with full statistical reliability (100%). This clade contains *C.parvum* and *C.hominis* as sister groups with a high bootstrap value of 85%. All the *C.hominis* isolates clustered together as did the *C.parvum* bovine genotype isolates with the exception of *C.parvum* isolated from C 115 as this isolate exhibited 3 nucleotide substitution. The NJ and ML trees for the COWP sequences support the diversity observations described previously (Xiao *et al.*, 2000b). The genetic relationship among *Cryptosporidium* parasites revealed by the COWP phylogenetic trees are largely congruent with the one produced by the analysis of the 18S rRNAgene.

Figure 5.4. Phylogeny of *Cryptosporidium* isolates by rooted NJ-tree based on COWP gene.

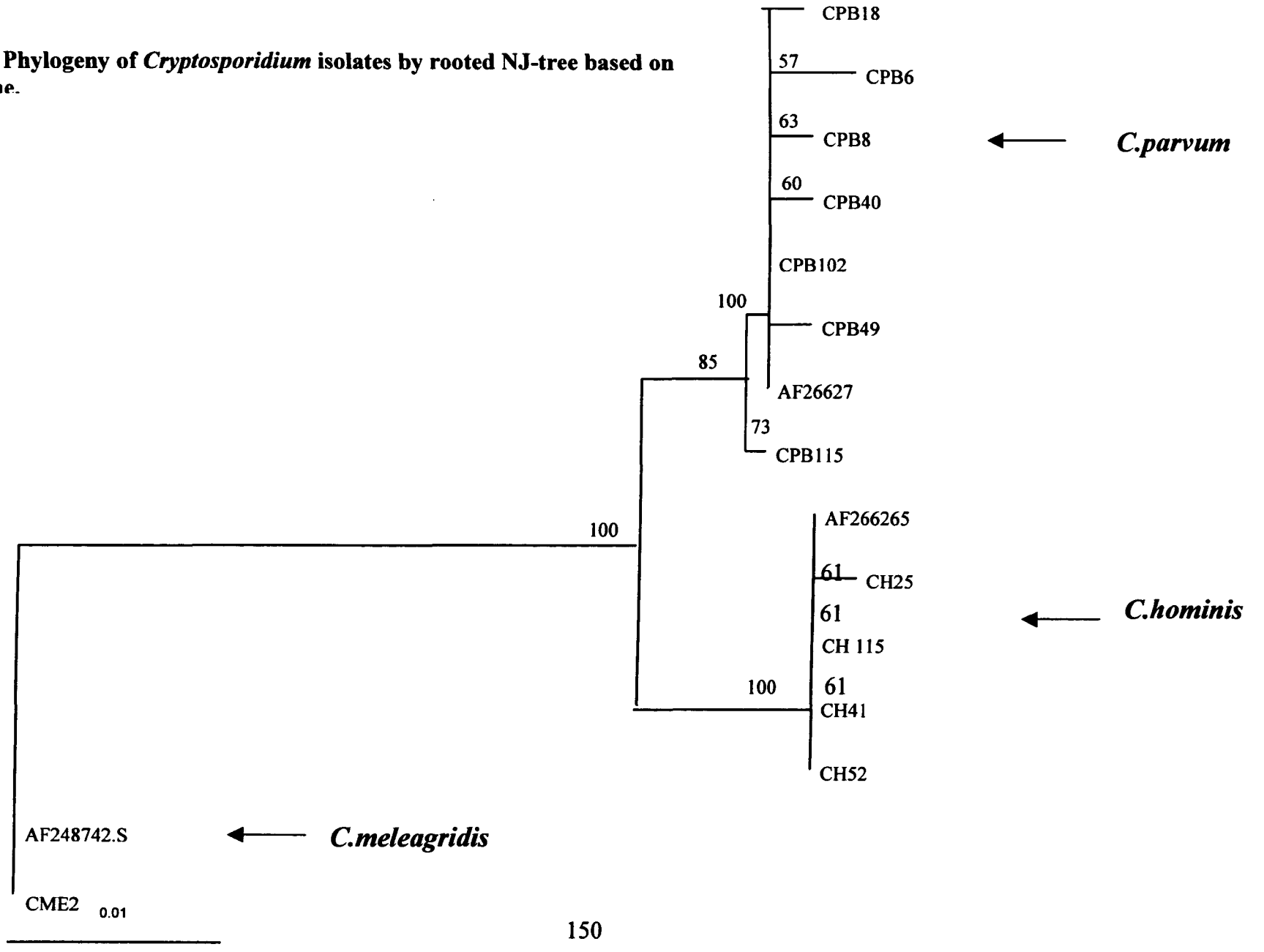


Figure 5.5. Phylogeny of *Cryptosporidium* isolates by a rooted ML tree based on COWP gene

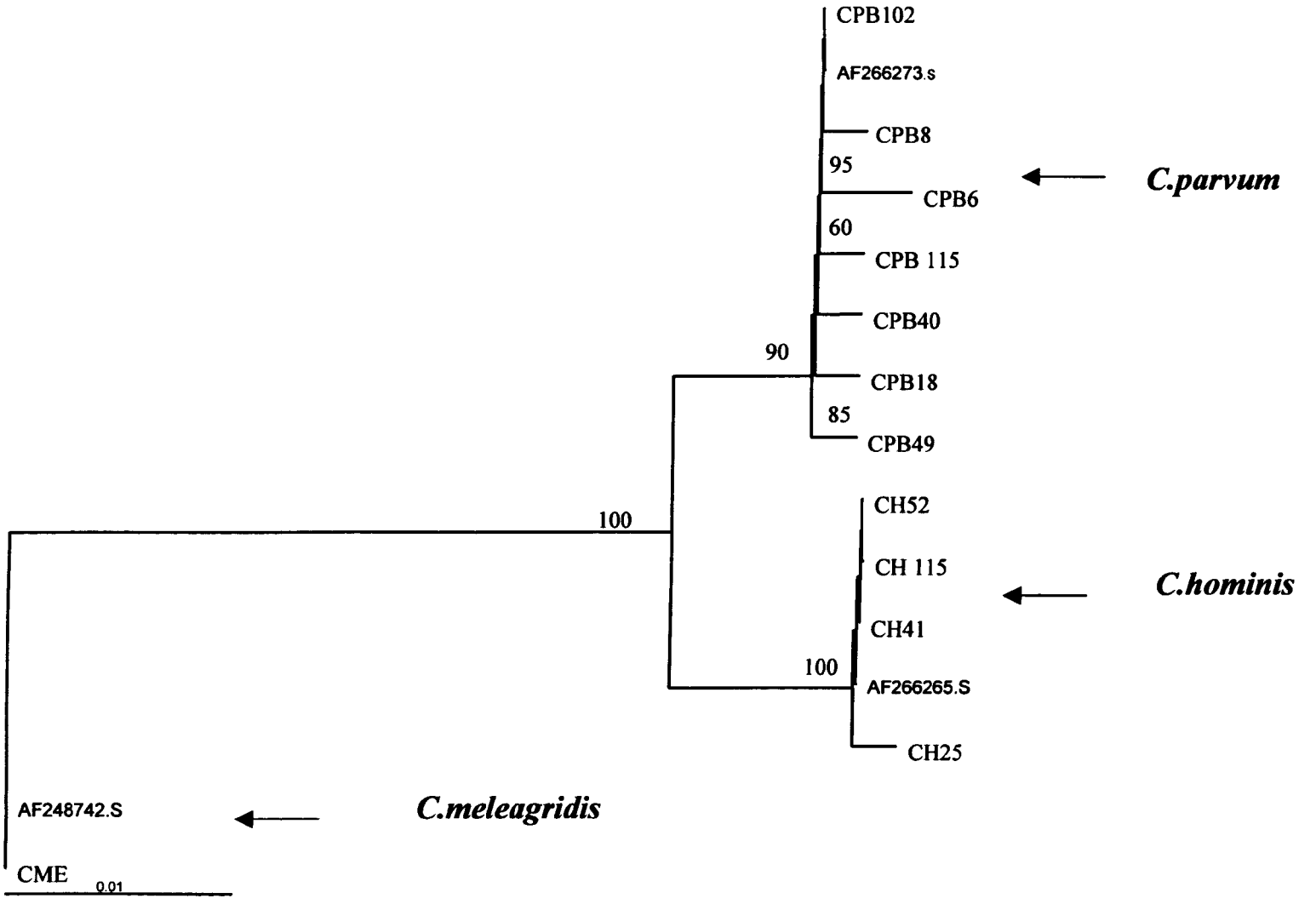


Table 5.5. Evolutionary genetic distance between selected *Cryptosporidium* isolatesbased on the COWP gene. (CPB 102 was used as consensus).

Distance method : Nucleotide: Kimura 2-parameter [Pairwise distances] Standard Error estimated by bootstrap method. Replications = 500 (Mega-2).

Species	Type		1	2	3	4	5	6	7	8	9	10	11	12
1-*C.parvum*	Bovine	CPB102	-											
2-*C.parvum*	Bovine	CPB115	0.2											
3-*C.parvum*	Bovine	CPB18	0.2	0.4										
4-*C.parvum*	Bovine	CPB40	0.2	0.4	0.4									
5-*C.parvum*	Bovine	CPB49	0.2	0.4	0.4	0.4								
6-*C.parvum*	Bovine	CPB6	0.4	0.6	0.6	0.6	0.6							
7-*C.parvum*	Bovine	CPB8	0.2	0.4	0.4	0.4	0.4	0.6						
8-*C.hominis*		CH25	1.6	1.9	1.8	1.9	1.9	2.1	1.8					
9-*C.hominis*		CH115	1.6	1.9	1.7	1.9	1.8	2.1	1.8	0.2				
10-*C.hominis*		CH41	1.4	1.6	1.7	1.6	1.6	1.9	1.6	0.2	0.3			
11-*C.hominis*		CH52	1.4	1.6	1.6	1.6	1.6	1.9	1.6	0.2	0.0	0.1		
12-*C.meleagridis*		CME26	3.5	3.3	3.7	3.7	3.7	4.0	3.7	4.0	3.7	3.7	3.7	-

Both nucleotide and amino acid alignments were used to assess the nucleotide substitutions and, mutation rates, the number of synonymous and non-synonymous substitution and the number of amino acid changes per site between the different sequences.

A total of 11 sequences, most of them defined by one or several point mutations were identified with the previous observation of the high diversity in the bovine genotype of *C.parvum* based on the 18S rRNA. This genotype also exhibited a high mutation rate of 7 with 7 singleton sites. The mutation rate per sequence (Theta/Seq) was 2.559 and the results of the neutrality test were not significant (Tajima D- value = - 1.55311, P=0.10). The results are shown in Table 5.6. The rate of nucleotide substitution is much higher at the third position in codons than at the first and second positions. Any nucleotide substitutions at the third position are silent and do not cause a change in the amino acid encoded. Indeed, the rate of non-synonymous substitution is generally much lower than that of synonymous and varies extensively from gene to gene.

Table 5.7 shows the silent and non-synonymous diversity within *Cryptosporidium* isolates. The sequence diversity was assessed using CPB 102 which showed 100% identity to the published strain 6 (Accession number AF 266273) in GenBank.

As observed from the Table 5.7, the synonymous substitution rate is much higher than the non-synonymous. There are two *C.parvum* bovine genotypes which exhibited two non-silent mutations. In CPB6 there is a cytosine to thymine substitute in the second position of the codon encoding Alanine (at 1316 nucleotide position based on the COWP complete gene) and this changes the amino acid Alanine to Valine (both Aliphatic amino acids). In CPB8 there is a thymine to adenine substitution in the first position of the codon encoding Serine (at 1156 nucleotide position) and this changes the amino acid to Threonine (both amino acids with hydroxyl containing side chain).

Figure 5.6 shows the summary of the variable sites for selected nucleotide sequences. These sequences were aligned according to CPB102.

The alignment of the amino acids sequences indicated that these amino acids units are all closely related (Figure 5.7).

Table 5. 6. Nucleotide diversity and mutation rate of *Cryptosporidium* isolates at the COWP locus.

Isolates	No of Seq	Total sites	S	Eta	Eta (s)	Theta/ Eta(S)	Pi	Theta /Seq	Theta /site	Tajima D-value	*P*
C.hominis	3	494	1	1	1	0.75	0.00101	0.545	0.00111	NA	NA
C.parvum	7	494	7	7	7	7.7	0.00405	2.559	0.00584	**-1.55311**	**0.10**

S =Number of polymorphic sites
Pi =Nucleotide diversity
Eta =Total Number of mutation
Eta(S) Total number of segregating sites
Theta/Eta(S)= mutation rate per singleton site
Theta/site=mutation rate per nucleotide site
Theta/Seq=mutation rate per sequence
TajimaD-value=neutrality test
P-value=level of significance differences between sequences

Table 5. 7. Synonymous and non-synonymous mutation rates for some of *Cryptosporidium* isolates based on the COWP gene.

Isolates	Species/genotypes	Synonymous	Non-synonymous	No of amino acid changes
CPB115	*C.parvum* Bovine	1	0	0
CPB18	*C.parvum*	1	0	0
CPB40	*C.parvum*	1	0	0
CPB49	*C.parvum*	1	0	0
CPB6	*C.parvum*	1	1	1
CPB8	*C.parvum*	0	1	1
CH41	*C.hominis*	7	0	0
CH115	*C.hominis*	7	0	0
CH25	*C.hominis*	8	0	0
CH52	*C.hominis*	7	0	0
CME26	*C.meleagridis*	16	1	1

Table 5.7. Shows the synonymous and non-synonymous changes from selected isolates based on the COWP gene. CPB 102 was used as a consensus; this isolate is similar to the published isolate 3 deposited in the GenBank accession number (AF 266273).

Figure 5.6. Summary of variable sites for selected sequences based on COWP gene (Number of variable sites = 28) Mega-2.

```
1-CP102   CATAAACGGC CTCTAAGTCA GTAATTCC
2-CP115   T.........  ..........  ..GT....
3-CP18    .T........  ..........  ........
4-CP40    .....G....  ..........  ........
5-CP49    ..........  .....G....  ........
6-CP6     ..........  T.........  A.......
7-CP8     ..........  ...C......  ........
8-CH25    ..CG..T.A.  .C......T.  ..GT....
9-CH41    ..C...T.A.  .C......T.  ..GT....
10-CH115  ..C...T.A.  .C......T.  ..GT....
10-CH52   ..C...T.A.  .C......T.  ..GT....
11-CME26  T...G..A.T .CT.T.TC.T .AGTCAAA
```

The CP 102 isolate (similar 100% to the published isolate AF 266273) was used as consensus sequences. Isolates from 1-6 are *C.parvum*, isolates from 8-10 are *C.hominis* and isolate 11 is *C.meleagridis*.

Figure 5.7. The amino acid sequences of selected isolates based on COWP gene.
(Bold positions show the polymorphic amino acid)

```
1-CP102   TMPEKSCPPG FVFSGKQCVQ SDTAPPNPEC PPGTILENGT CKLIQQIDTV CPSGFVEEGN RCVQYLPANK ICPPGFNLSG  [ 80]
2-CP115   .......... .......... .......... .......... .......... .......... .......... ..........
3-CP18    .......... .......... .......... .......... .......... .......... .......... ..........
4-CP40    .......... .......... .......... .......... .......... .......... .......... ..........
5-CP49    .......... .......... .......... .......... .......... .......... .......... ..........
6-CP6     .......... .......... .......... .......... .......... .......... .......... ..........
7-CP8     .......... .......... .......... .......... .......... .......... .......... .........T.
8-CH25    .......... .......... .......... .......... .......... .......... .......... ..........
9-CH41    .......... .......... .......... .......... .......... .......... .......... ..........
10-CH52   .......... .......... .......... .......... .......... .......... .......... ..........
11-CME26  .......... .......... .......... .......... .......... .......... .......... ..........

1-CP102   QQCMAPESAE LESTCPPNSI FENGKCKVIK NIDMVCPPGY TDSGDDCVLY VAPAKECPPN FILQGLQCIQ TSSAPTQPVC  [160]
2-CP115   .......... .......... .......... .......... .......... .......... .......... ..........
3-CP18    .......... .......... .......... .......... .......... .......... .......... ..........
4-CP40    .......... .......... .......... .......... .......... .......... .......... ..........
5-CP49    .......... .......... .......... .......... .......... .......... .......... ..........
6-CP6     .......... .......... .......... .......... .......... .V........ .......... ..........
7-CP8     .......... .......... .......... .......... .......... .......... .......... ..........
8-CH25    .......... .......... .......... .......... .......... .......... .......... ..........
9-CH41    .......... .......... .......... .......... .......... .......... .......... ..........
10-CH52   .......... .......... .......... .......... .......... .......... .......... ..........
11-CME26  .......... .......... .......... .......... .......... .......... .......... ..........
```

5.3.5. Phylogeny and nucleotide diversity for gp 15/45/60 gene

The gp 15/45/60 nucleotide and deduced amino acid sequences from all isolates studied in this locus revealed extensive polymorphisms. Comparison of these sequences revealed several specific polymorphic as well as conserved regions. Sequence alignment revealed extensive differences in the nucleotide sequences through the entire length of the fragment. The levels of intra-specific variation between the *C.hominis* isolates were greater (61.3-68.4%) than the levels of interspecific variation between *C.hominis* and *C.parvum* (63.5-73.9%) (Table 5.9).

Within *C.parvum* alleles, allelic IIa contained all *C.parvum* isolates and two isolates from *C.hominis*, all the isolates within this allelic exhibited the same subgenotype. In contrast to the limited genetic diversity in *C.parvum*, the *C.hominis* alleles Ib and Id exhibited the same sub-genotype within each allelic group with the exception of the Ia allele group which showed different subgenotypes. *C.parvum* isolated from C115 displayed a hybrid pattern of both *C.hominis* Ic and *C.parvum* bovine genotype IIa specific SNPS. This mixed lineage could have been derived by sexual recombination between *C.hominis* carried the Ic allelic group and *C.parvum* carried the IIa allelic group as hypothesized previously by Leav and Others (2002).

The relatedness and the polymorphisms of the *Cryptosporidium* isolates at this locus is illustrated in Figures 5.8 and 5.9. The *C.parvum* and *C.hominis* isolates formed 4 different clades with full statistical reliability. In the NJ method the *C.hominis* allelic IIa genotypes placed as a sister group with the *C.parvum* isolates. These isolates were placed at the earliest branching point followed by *C.hominis* Ia allelic group. The *C.hominis* Ib isolate was clearly placed together within *C.hominis* Id isolates as a sister group. The *C.parvum* allele family Ic appears phylogentically related to the *C.hominis* allele family Id, whereas *C.parvum* allele family IIa is related to the *C.hominis* allele family Ia. The pattern of sequence diversity in the Ic allele family is also different from that in all other *C.parvum* and *C.hominis* allele families.

Bootstrap analysis of the distance data provided strong support (100%) for the placement of the Ic allele family on the separate unique branching lineages and for the grouping together of Ib and Id allelic groups (99%). Strong support was also observed for the clustering of the *C.parvum* bovine isolates with each other (100%). The relatedness of these isolates based on the ML method is illustrated in Figure 5.9.

The degree of nucleic acid sequence polymorphisms displayed by the gp 15/45/60 alleles is surprising and unprecedented; not only are the various *C.hominis* allelic nucleotide sequences very different from *C.parvum* (bovine genotype) sequences, they are also very different from one another. All of the *C.hominis* isolates genotyped at the gp 15/45/60 locus have also been genotyped at other loci. For instance, isolates CH 52 and CH 41, CH 7 and CH 115 and ChH25 representing the gp 15/45/60 Ia, Ib, and Id alleles respectively were genotyped by PCR-RFLP and DNA sequencing at 18S rRNA locus. These isolates displayed identical *C.hominis* 18S rRNA patterns (99.2% identity). We also genotyped these isolates at the COWP locus and found all had the previously described species specific sequence (99.8-100% identity). Table 5.8 shows the different isolates studied at the different genetic loci.

Figure 5.8.Phylogeny of *Cryptosporidium* isolates by a rotted NJ-tree based on gp 15/45/60 gene.

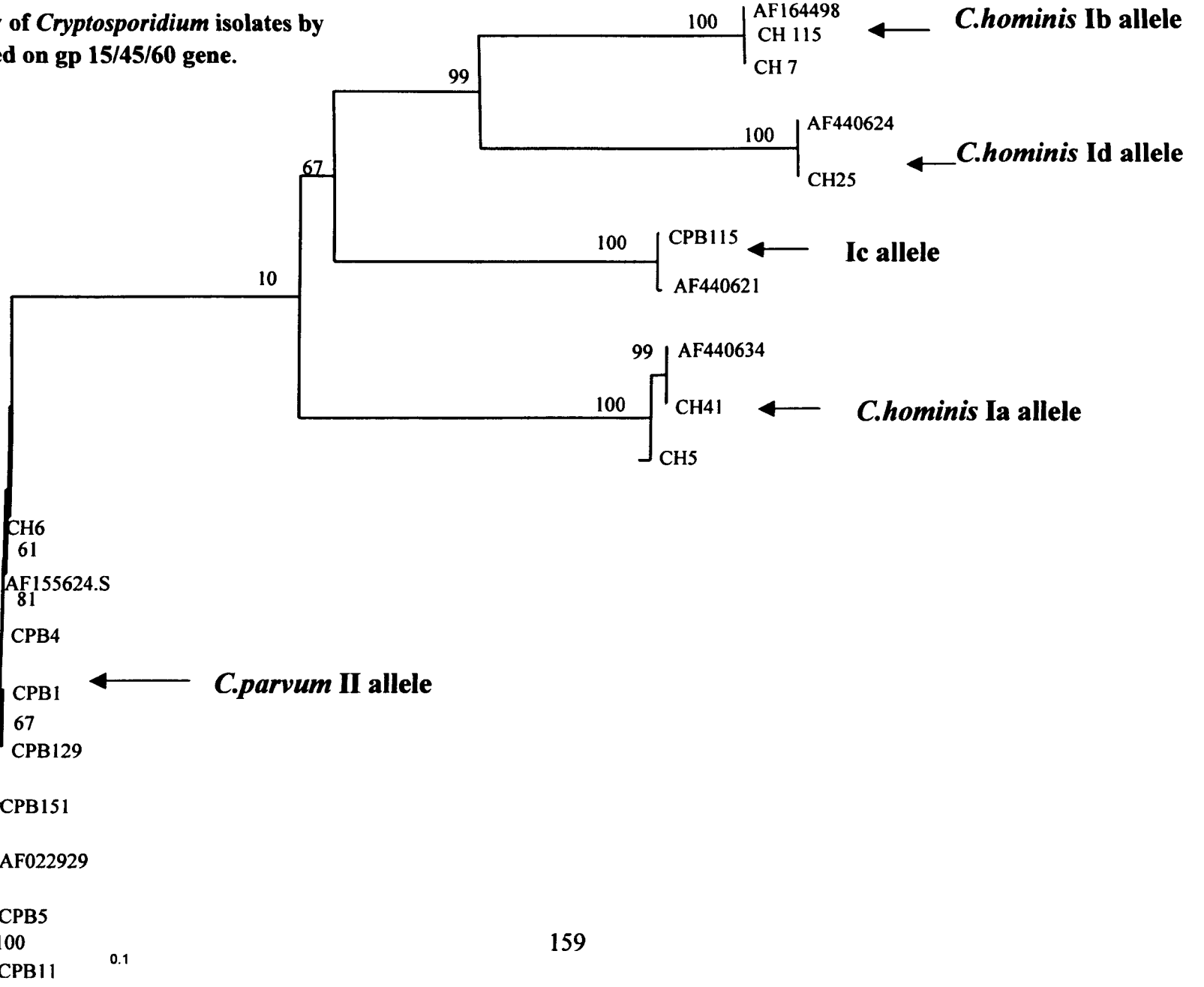


Figure 5.9. Phylogeny of *Cryptosporidium* isolates by a rooted ML-tree based on gp 15/45/60 gene

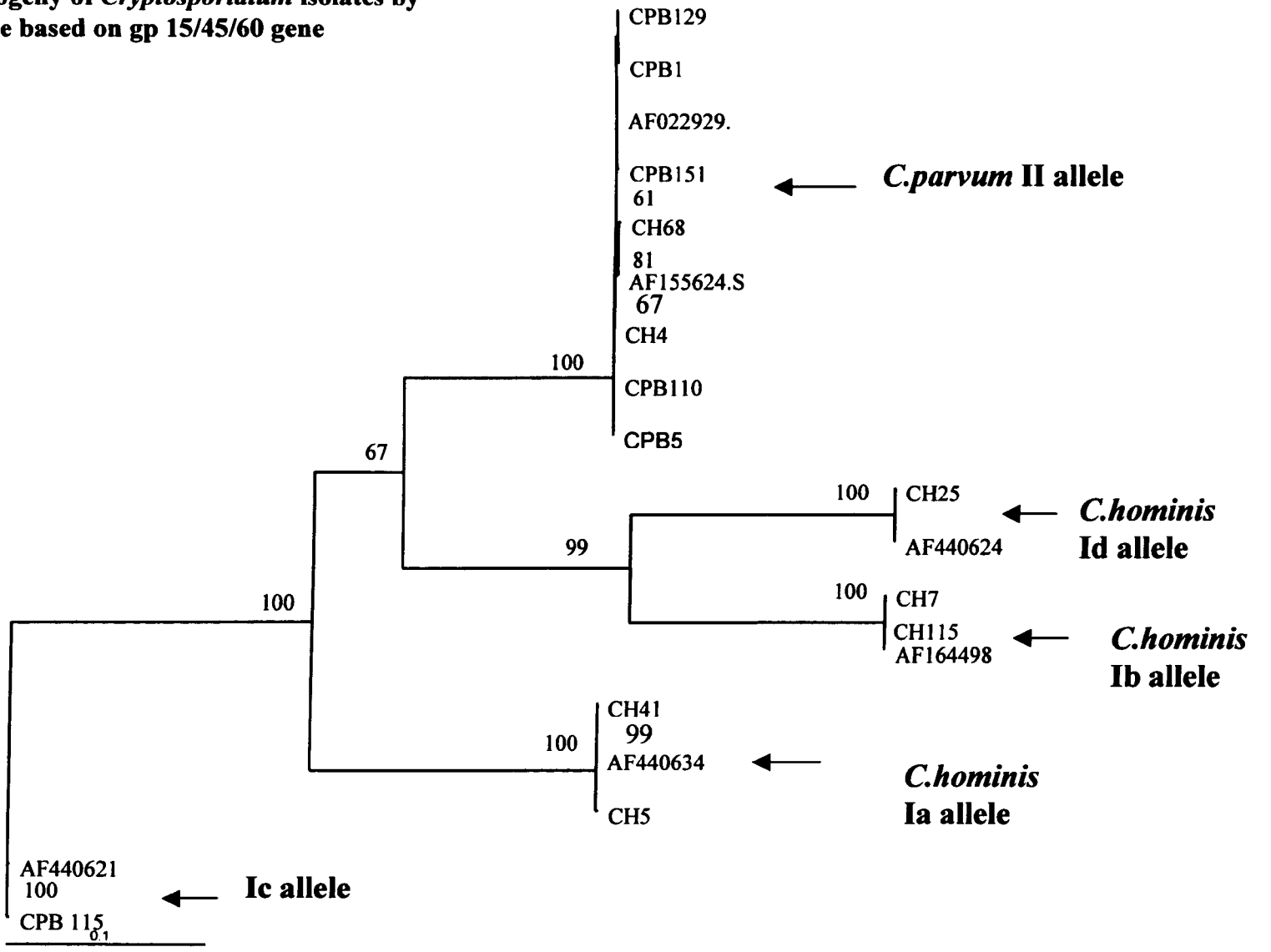


Table 5.8. The results of species identified from selected isolates based on the different loci examined (18S rRNA, COWP, and gp 15/45/60).

Isolates	Species	Loci		gp15/45/60
		18S-rRNA	COWP	
CH41	*C.hominis*	*C.hominis*	*C.hominis*	Ia
CH52	*C.hominis*	*C.hominis*	*C.hominis*	Ia
CPB115	*C.parvum*	Chimera		Ic Chimeric
C H115	*C.hominis*	Chimera		Ib
CH25	*C.hominis*	*C.hominis*	*C.hominis*	Id
CH7	*C.hominis*	*C.hominis*	*C.hominis*	Ib
CH68	*C.hominis*	*C.hominis*	*C.hominis*	II
CH4	*C.parvum bovine*	*C.parvum*	*C.parvum*	II
CPB129	*C.parvum* bovine	*C.parvum*	*C.parvum*	II
CPB1	*C.parvum* bovine	*C.parvum*	*C.parvum*	II
CPB151	*C.parvum* bovine	*C.parvum*	*C.parvum*	II
CPB5	*C.parvum* bovine	*C.pavum*	*C.parvum*	II
CPB110	*C.parvum* bovine	*C.parvum*	*C.parvum*	II

Table 5.8. Shows the results of sequence analysis for selected isolates based on the 18S-rRNA, COWP, and gp 15/45/60 loci. C 115 demonstrated both species *C.parvum* and *C.hominis* at the 18S-rRNA and COWP loci. In the gp 15/45/60 locus *C.hominis* from this isolate exhibited the Ib allele family and the *C.parvum* exhibited the Ic allele family after the sequence analysis for this isolate.

Each complete DNA and deduced protein sequence was compared with each of the other complete sequences and the percentages of identical nucleotide and amino acids residues were tabulated for each pairwise comparison (Table 5.9). All the bovine *C.parvum* isolates (with the exception of *C.parvum* isolated from C 115) and two of the *C.hominis* isolates were identical or very nearly identical to each other. This homogeneous sequence family defined a single gp15/45/60 allele. This gene sequence derived from *C.hominis* isolates, however, displayed extensive polymorphism. Further analysis of the deduced gp 15/45/60 amino acid sequence has revealed many conserved and polymorphic features of the protein that are presumably important to its structure and/or function (Figure 5.10).

Table 5.9. The sequence identities that define the five gp15/45/60 alleles group from selected isolates.

Allelic isolation number	Ia		Ib		Ic	Id	IIa						
	CH 52	CH 41	CH7	CH115	CP115	CH25	CPB129	CPB110	CH68	CPB1	CH4	CPB5	CPB151
Ia CH52	**100**	**99.9**											
Ia CH41	**99.9**	**100**											
Ib CH7	68.5	68.4	**100**	**99.8**									
Ib CH115	68.6	68.4	**99.8**	**100**									
Ic CPB115	60.7	60.7	64.8	64.5	**100**								
Id CH25	61.5	61.3	61.6	61.6	64.4	**100**							
II CPB129	68.5	68.5	65.7	65.7	73.9	64.6	**100**	**99.9**	**99.1**	**100**	**99.2**	**99.9**	**99.1**
CPB110	68.5	68.5	65.7	65.7	73.9	64.6	**99.9**	**100**	**99.1**	**99.9**	**99.4**	**100**	**99.2**
CH68	69.7	68.3	64.6	64.6	73.8	64.3	**99.1**	**99.1**	**100**	**99.1**	**99.2**	**99.1**	**98.2**
CPB1	68.5	68.5	65.7	65.7	73.9	64.6	**100**	**99.9**	**99.1**	**100**	**99.2**	**99.9**	**99.1**
CH4	69.2	69.2	66.6	66.4	73.9	64.3	**99.2**	**99.4**	**99.2**	**99.2**	**100**	**99.4**	**98.5**
CPB5	68.5	68.5	65.7	65	73.9	64.6	**99.9**	**100**	**99.1**	**99.9**	**99.4**	**100**	**99.2**
CPB151	70.9	71	66.4	66.3	73.0	63.5	**99**	**99.2**	**98.2**	**99.1**	**98.5**	**99.2**	**100**

Table 5.9 demonstrate the percentage identities in the nucleotide sequences from selected isolates based on the gp 15/45/60 locus. Intra-allelic sequence comparisons are shown in boldface type.

The deduced amino acid sequence of the gp15/45/60 locus contains an N-terminal signal sequence and a C-terminal glycophosphatidyl inositol (GPI) anchor site. A striking feature of the gene is the polyserine domain present in the N-terminal region of the mature polypeptide which varies in length between 9-24 amino acids.

The original IIa isolate sequence is the reference sequence to which all the others are compared. The N –terminal 5 amino acids, DVSVE were conserved in all isolates. The polyserine tract was conserved but varied in length among isolates. Downstream of the polyserine tract, all isolates contained a hyper-variable region characterized by numerous SAAPs. The last and, perhaps, most significant feature, is the putative proteolytic processing site apparently used to generate the mature gp15 and gp45 products from the gp15 precursor which was conserved among all isolates.

The *C.parvum* Ic allele family isolates displayed SAAPS that were nearly identical to those present in the deduced genotype IIa and Ic protein sequences. Figure 5.11 shows a comparison of the deduced amino acid sequences of the *C.parvum* Ic allele (C 115 isolate) with the genotype II allele.

Figure 5.10. The amino acid motifs in the different allelic group among the *C.hominis* and *C.parvum* isolates.

```
IIa   DVPVEGSSSS SSSSSSSS SSSSSSSSSS STSTVAPAN
Id    DVSVESSSSS S-----SS SSSSSSSSSS ST-TVAPAS
Ia    DVSVESSSSS SSSSS-SS SSSSSSSSSS TS-TVAPAP
Ic    DVSVE----- -------- -SSSSSSSSS TT-TPAPAP
Ib    DVSVE----S S------S SSSSSSSSSS TT-TPAPAP

IIa   ---KAR-- -TGEDA---E GSQ------- ------DSSG
Id    NFTKAR-- -TGEDTGRSE GSQGSEEHQD GEDDSSDSSG
Ia    K--KER-- -TVEGG--TE GKN------- --EE--SSPG
Ic    K--KVR-- ESEEGKN-SE DSQ------- ----TPASPG
Ib    KKAFTREA DGGEEKN-NE ESQ------- ----TPASPG

IIa   TE---A S-GSQ----- GSEE-EGSE DDG-QTSFT AASQ------
Id    GS---V G-GTESGSAG GKNE-EDSS SSGGAQDFT GSGGTAEGAT
Ia    SEEQ-D G-GKEDG--- GKENGEGDT VDG--E-QT GSGS------
Ic    S----G SQDS------ SKGD---EV VDG------ GASG------
Ib    SGGVSE GQDTQGG--- SKGDAEEGT EDNEQADE- SATQ------

IIa   -------PTT PAQSEG-ATT E-TIEATPKE ECGTSFVMWF GEGTPAATLK
Id    QSEATASQGA PSQGSD-KTT EST-QTTPKE ECGTSFVMWF GEGTPVATLK
Ia    -------QVT PSGSAG-TAT ESTATTTPKE ECGTSFVMWF EKGTPVATLK
Ic    -------PST PTQATE-KEP E-TPESTPKE ECGTSFIMWF GEGTPATTLK
Ib    -------PST PFTGQGSVKT EST-ETTPKE KCGTSFVMWF GQGVPVATLK

IIa   C--GAYTIVY APIKDQFTTD PAPRYIS--G EVTSVTFEK- SD-NTVKIKV
Id    CFTGGYTIVY APVKDQ--AN PAPRYIS--G EVKNVSFQKE SD-NTIKIKV
Ia    C--GDYTIVY APIKDQ--TD PAPRYIS--G EVTSVSFEK- SES-TVTIKV
Ic    C--GGYTIVY APEKDN--KE PAPRYIS--G DVKDVTFEK- GQGNTVKIKV
Ib    C--GDYTMVY APEKDK--TD PAPRYISFTG EVTTVTFDK- QES-TVTIKV

IIa   NGQDFSTLSA NSSSPTE--N GGSAG--QAS SRSRRFTSLS EETSE-AAAT
Id    DGQDFSTLSA SSSSPTEFTN KGESGN-QVE SRSRR--SLT EETSE--TAT
Ia    NGKEFSTLSA NSSSPTK--D NGESSDSQVQ SRSRR--SLA EENGE-TVAT
Ic    DGKEFSTLSS SSSNPTE--N NGSAG--QVA SRSRR--SLS EENSE-TAAT
Ib    NNVEFGTLST SSSKPTE--N KGESSD-QVG SRSRR--SLT EETSEFTTAT

IIa   VDLFAFTLDG GKRIEVAVPN VEDASKRDKY SLV--ADDKP FYTGAN
Id    VDLFAFTLNG GKRIEVAVPN AEETSKRDKY SLVFTADDSA FYTGKN
Ia    VDLFAFTLDG GRRIEVAVPK DENADKRSEY SLV--ADDKP FYTGAN
Ic    VDLFAFTLDG GQRIEVAVPS VEDATKRDKY SLV--ANGKP FYTGAN
Ib    VDLFAFTLDG GKRIEVAVPS DEDVSKRNKY SLV--ANDKT FYTGAN
```

(The blue colour shows the polyserine tract and the red is the cleavage site between gp15 and gp45, dashes denote nucleotide deletions)

Figure 5.11. The comparison of SAAPs in the gp 15/45/60 of allelic Ic with those of allele group IIa . These sequences were aligned using Clustal X.(The highlighted sequence shows the similarity between the two allelic)

```
CP115(Ic)      DVSVE.....  ........  .SSSSSSSSS TTTPAPAPKK VRESEEGKNS
CPB151(IIa)    DVPVEGSSSS SSSSSSSS SSSSSSSSS STSTVAPANK ARTGEDAEGS

CP115(Ic)      EDSQTPASPG S.GSQDSSKG DEVVDG..GA SGPSTPTQAT EKEPETPEST
CPB151(IIa)    QDSSGTEASG SQGSEEEGSE DDGQTSFTAA SQPTTPAQSE GATTETIEAT

CP115(Ic)      PKEECGTSFI MWFGEGTPAT TLKCGGYTIV YAPEKDN..K EPAPRYISGD
CPB151(IIa)    PKEECGTSFV MWFGEGTPAA TLKCGAYTIV YAPIKDQFTT DPAPRYISGE

CP115(Ic)      VKDVTFEKGQ GNTVKIKVDG KEFSTLSSSS SNPTENNGSA GQVASRSRR.
CPB151(IIa)    VTSVTFEKSD .NTVKIKVNG QDFSTLSANS SSPTENGGSA GQASSRSRRF

CP115(Ic)      .SLSEENSET AATVDLFAFT LDGGQRIEVA VPSVEDATKR DKYSLVANGK
CPB151(IIa)    TSLSEETSEA AATVDLFAFT LDGGKRIEVA VPNVEDASKR DKYSLVADDK

CP115(Ic)      PFYTGAN
CP151(IIa)     PFYTGAN
```

5.4. DISCUSSION

In this chapter analysis of DNA sequences and phylogenetic analysis have confirmed the identities of the isolates recovered. This study is the first to report on human infection with *Cryptosporidium* spp identified using microscopy and their molecular analysis in Saudi Arabia. This study is also in agreement with other reports from UK (McLauchlin *et al.*, 2000) and France (Guyot *et al.*, 2001) that demonstrates the high prevalence of zoonotic species of *Cryptosporidium* including *C.parvum* Bovine genotype in human infection.

In pairwise comparisons of 18S rRNA sequences, the data for some *Cryptosporidium* isolates revealed interspecies differences significantly higher than intraspecies differences; for example, the similarity between two bovine isolates of *C.parvum* was 99% whereas the similarity between *C.parvum* and *C.hominis* was 98.1%, *C.parvum* and *C.meleagridis* 97.3% and *C.parvum* and *C.muris* 86.8%. These results compare well with the level of similarity between avian *Eimeria* species whose phylogenetic relationships have been well characterized (Barta *et al.*, 1997). Heterogeneity appeared to be in the amplification of gene B from two *C.parvum* isolates (CPB129 and CPB40). The 18S rRNA gene exhibits intraspecific heterogeneity occurring as 4 copies of type A and one copy of type B-18S rDNA units per haploid genome (Le Blancq *et al.*, 1997; Xiao *et al.*, 1999b). Unlike malarial parasites the differences between the heterogeneous 18S rRNA copies in *Cryptosporidium* parasites is small (Li *et al.*, 1994) thus the impact of this heterogeneity appears to have little effect as described from the results of phylogenetic analysis (Xiao *et al.*, 1999a). In addition it also presents no significant challenge to 18S rRNA based species and genotype differentiation PCR-RFLP tool (Xiao *et al.*, 1998).

Various *Cryptosporidium* species have extensive sequence polymorphisms in the COWP gene, which seems to reflect the genetic relatedness of different *Cryptosporidium* parasites. Certain *Cryptosporidium* parasites were more related to each other than others as reflected in the number of base pair differences among them and the genetic distances calculated.

The amino acid substitution in this gene caused by the fact that substitution occurs more often with amino acids that are similar in terms of biochemical properties such as polarity and size than between dissimilar amino acids. In other words, amino acid substitution is generally far from random and it may occur quite often with similar amino acids.

The largest intra-genotypic variation was within the *C.parvum* bovine isolates. Single nucleotide substitution occurred along the lengths of both, the 18S rRNA and COWP loci. On the other hand; there appears to be a great degree of diversity among *C.hominis* isolates. This genetic diversity suggests that such genes are under high selection pressure.

During the mid to late 1980s it was discovered that the 18S rRNA gene sequence had several properties that render them useful in phylogenetic analysis (Olsen & Woese, 1993). The 18S rRNA genes are of an intermediate size relative to the large and 5.8 genes and are among the slowest evolving sequence found throughout living organisms (Carreno *et al.*, 2001). In contrast, PCR-RFLP analysis and sequencing of the 18S rRNA gene together form a useful tool for differentiating *Cryptosporidium* isolates the variation in the gene being adequate for the identification of the species and sub-species of the parasites. The ability to amplify the 18S rRNA fragment from different species and genotypes of the organisms using one set of primers make it appropriate for screening where the species types of *Cryptosporidia* occurring are unknown (Gatei *et al.*, 2003).

The COWP gene also provides a useful alternative target for molecular taxonomy and phylogenetic analysis of *Cryptosporidium* parasites. The COWP gene was recently sequenced and analyzed in *C.parvum* and its utility in distinguishing *Cryptosporidium* spp was indicated by Spano and Colleagues (1997b) who compared a part of the COWP gene sequence among several isolates of *C.parvum* and in *C.wrairi*.

The results of the phylogenetic analysis based on the 18S rRNA and COWP loci are in agreement with biologic differences between the two groups of *Cryptosporidium* parasites. Biologically the two *Cryptosporidium* groups have different predilections for sites of infection, *C.parvum*, *C.hominis*, and *C.meleagridis* each infect the small intestine whereas *C.muris* infects the stomach (Morgan *et al.*, 1999d).

The present study also demonstrates a remarkable degree of genetic heterogeneity at the gp 15/45/60 locus among *Cryptosporidium* isolates. These finding confirmed and extended the observation of previous studies (Strong *et al.*, 2000; Leav *et al.*, 2002; O'Connor *et al.*, 2002; Alves *et al.*, 2003). An important feature of this gene its high degree of sequence polymorphism, particularly among *C.hominis* isolates, which is far greater than any other *Cryptosporidium* genetic loci examined to date (Sualiman *et al.*, 2001; Leav *et al.*, 2002). The majority of the genes previously studied encode cytoplasmic proteins however, even genes that encode cell surface-associated proteins such as TRAP-C1 have shown remarkably little sequence variation within or even between the two species (Spano *et al.*, 1998).

Our study demonstrated the occurrence of mixed infection of *C.parvum* bovine genotype and *C.hominis* in one isolate (C115). Although humans are susceptible to both types, surprisingly only a few mixed infections have been reported. For instance, using RFLP markers only one mixed infection was reported among 49 human cases from a cryptosporidiosis outbreak in the UK (McLauchlin *et al.*, 1999) and no mixed infections were reported among 50 human isolates from various outbreaks and sporadic cases in the USA (Sulaiman *et al.*, 1998). In contrast, a recent survey using micro-and mini-satellite length polymorphisms found 1/2% *C.hominis* and *C.parvum* bovine genotype mixed infections among 135 human cryptosporidiosis cases from Scotland (Mallon *et al.*, 2003).

The *C.parvum* isolated from C 115 was genotyped as Ic at the gp 15/45/60 locus, this allelic type was apparently also the dominant type in South Africa (Leav *et al* ., 2002) and 5/16 of *C.parvum* isolates from Portuguese AIDS patients had this allele (Alves *et al.*, 2003). However, allele family Ic has never been found among over 500 bovine *C.parvum* isolates analysed thus far in the USA, and UK (Glaberman *et*

al., 2002; Alves *et al.*, 2003; Peng *et al.*, 2003). *C.parvum* from isolate C 115 was genotyped as bovine genotype based on the PCR-RFLP and DNA sequence analysis based on the 18S rRNA and the COWP loci and, moreover, the *C.hominis* from the same isolate was genotyped as *C.hominis* based on the three loci and this might suggest that this mixed genotype lineage *C.parvum* Ic was derived by sexual recombination between the two species as hypothesized previously and emerge within the population (Strong *et al.*, 2000; Leav *et al.*, 2002). It was previously suggested that gp 15/56/60 allele family Ic resulted as a recombination between the *C.parvum* allele IIa and *C.hominis* Ic (Leav *et al.*, 2002).

These observation strongly suggest that progenitors of the *C.parvum* allele family Ic at gp15/45/60 could have been derived by intragenic recombination between parasites carrying a prototypal Ic allele which donated the 5' end two thirds sequence and isolates carrying IIa allele which provided the respective 3' ends of the sequence, thus the *C.parvum* parasite isolate has the genetic feature of both clonal lineage and a recombinant haplotype presumably derived by sexual recombination between them.

The recombinant haplotype appear to have arisen through both of the usual mechanisms that create the genetic diversity during sexual reproduction; i.e. independant assortment of the chromosome and crossing over as both the 18S rRNA and COWP genes segregated independently. Although meiotic recombination between *C.parvum* bovine parasites has recently been demonstrated experimentally in an animal host (Feng *et al.*, 2002), no similar data are available regarding sexual recombination between the two parasites lineages. This is thought to be an exceedingly rare or nonexistent event in natural populations, as isolates manifesting recombinant haplotypes have not been previously detected (McLauchlin *et al.*, 1999).

Within the phylum apicomplexa, recombination has been observed in the laboratory with the malaria parasite *Plasmodium falciparum* (Walliker *et al.*, 1987) and the enteric coccidian *Eimeria* (Sutton *et al.*, 1986) and *Toxoplasma gondii* (Sibley *et al.*, 1992). In *P.falciparum* field studies have shown that recombination between

genetically distinct parasites also occurs in nature (Babiker *et al.*, 1994; Conway *et al.*, 1999).

The observation that the gp15/45/60 gene is highly polymorphic among *C.hominis* isolates but with little variation among bovine genotype isolates suggested the possibility that control of expression of this gene could also differ between the two isolates (O'Connor *et al.*, 2002). These data have also raised the possibility that *Cryptosporidium* similar to other apicomplexans might be capable of antigenenic variation (Saul, 1999). It is unclear if the observed polymorphisms translate into functional differences such as recognition of different receptors or if the variation is a reflection of immune selection pressure or both (Strong *et al.*, 2000). However O'Connor and Others (2002) observed that the polymorphisms at this locus are not generated through the use of multiple gene copies. The level of gp15/45/60 genetic and amino acid diversity suggests that antigenic differences should also occur (Sestak *et al.*, 2002).

The finding that two isolates which had been demonstrated to be *C.hominis* based on 18S rRNA and COWP genes had IIa allele is not surprising in the light of the biology of *Cryptosporidium*. It remains to be elucidated why *C.parvum* isolates do not exhibit the same high level of polymorphisms as that among *C.hominis*. It is especially important to know whether genotypes of *Cryptosporidium* are stable, indicative of clonal propagation or unstable due to frequent genetic recombination although the population structure of *Cryptosporidium* has not been conclusively determined (Mallon *et al.*, 2003).

CHAPTER SIX

INVESTIGATION OF ADULT-SERA FOR THE PRESENCE OF ANTI-*CRYPTOSPORIDIUM* ANTIBODIES

6.1. INTRODUCTION

The spectrum and severity of cryptosporidial disease is directly linked to the immune status of the host, in immune competent humans the immune system controls *Cryptosporidium* and this is demonstrated by the usually self-limiting nature of the infection. In addition, the immune system may provide protection resulting in less severe disease upon re-exposure (Smith *et al.*, 2000). Random serology–based studies in humans and animals have suggested that infection with this organism is common during a life time (Tzipori & Campbell, 1981; Ungar & Nash, 1986). This suggests that persons with pre-existing antibodies may be resistant to infection and clinical disease.

Several studies support a role for IFN-γ in mediating the initial resistance to *C.parvum*, although the mechanism by which this cytokine imparts resistance is unclear(Mead *et al.*,1988; Chen *et al.*, 1993; Harp *et al.*,1994). It is also generally agreed that $CD4^+$ T lymphocytes are required for the resolution of both acute and chronic cryptosporidios . However, the effector mechanism is again unclear (Wyatt *et al.*, 2002). In contrast, immunodeficent persons usually develop persistent infections of greater severity and exhibit higher mortality rates. Several studies have indicated that number $CD4^+$ T lymphocytes are directly related to resolution of cryptosporidiosis (Ungar *et al.*, 1991; Aguirre *et al.*, 1994; Perryman *et al.*, 1994). Studies showing the importance also of INFγ, IL 12, $CD4^+$, and $CD8^+$ T cells in clearing primary cryptosporidiosis followed by the development of subsequent resistance to *Cryptosporidium* infection have also been reported (Pasquali *et al.*, 1997; Abrahamsen, 1998; Wyatt *et al.*, 2002).

A humoral response to infection has been demonstrated in a wide range of mammals including both immunologically healthy and immunocompromised humans (Fayer

&Ungar, 1986). Although specific Immunoglobulines IgA, IgG, IgE, and IgM antibodies to sporozoites and oocysts have been identified (Abrahamsen, 1998), these antibodies are usually expressed in response to specific low molecular mass parasite-proteins of 17 and 27 KDa.

As mentioned previously in chapter 3, the detection of *Cryptosporidium* oocysts from faecal samples is the method used in most general laboratories for diagnosis of cryptosporidiosis. Serological methods can be used to identify past exposure to infection in individuals by demonstrating the antibody response to specific antigens but will only provide retrospective diagnosis. Although, serological methods may not be usefull for routine diagnosis of infection, they are useful as epidemiological tools to determine the sero-prevalence of infection in communities.

The purpose of work presented in this chapter is to survey representatives healthy adult population from Jeddah city, Saudi Arabia for the presence of antibodies to *Cryptosporidium* spp in order better to define the adult-related frequency of infection in the area. Additionally, risk factors that might be associated with seropositivity for *Cryptosporidium* antibodies were investigated.

6. 2. MATERIAL AND METHODS

6.2.1. Study population

A formal letter from the Ministry of Health was obtained to ask two major public hospitals in the area to participate in the study. One hundred and thirty conventional out-patients representing healthy young adult males and females aged from 18-30 years were recruited to participate in the study. A total of 65 participants were selected randomly from each hospital by the blood bank technician. The purpose of the study was explained to the head of the hospital and the chairman of the blood bank for each hospital. The study was explained to the participants orally (culturally accepted) and/or by given them the information sheet (Appendix 12). All the participants agreed to participate in the study and signed an informed consent letter

(Appendix 15) and completed a 2-page questionnaire (Appendix 14) that asked about age, sex, whether they have recently suffered from diarrhoea and if so what was its duration. They were also asked if they had suffered from any other episode of diarrhoea over the last 12 months. They were asked about the numbers of adults and children living in the household and if any of them had had diarrhoea in the last 14 days, they were also asked if they had any close contact with domestic animals and what was the source of their drinking water supply. The information sheet, consent letter and questionnaire were translated into Arabic (Appendix 11a, 12a and 13a).

Blood was taken into plastic tubes and allowed to clot. Following this, the sera were separated carefully and frozen at –20°C. The sera were shipped frozen to the LSTM. These samples were collected between November 2001 and April 2002.

6.2.2. *Cryptosporidium* antigen

The *Cryptosporidium* antigen was provided by the Public Health Laboratory Service (PHLS), *Cryptosporidium* Reference Unit, Swansea, PHL Singleton Hospital, Wales UK. *C.Parvum* oocysts suspension were obtained from Pleasant Hill Farm, Iowa, USA.

6.2.3. Positive and negative controls

Positive control serum was collected from a British patient with confirmed cryptosporidiosis who had strong serological response to the two antigens. A negative control serum was obtained from a British person whose stool was negative for oocysts and who had no detectable antibody to these antigens. Positive and negative controls were included on each blot. Positive and negative controls were supplied by the PHLS, Swansea, UK.

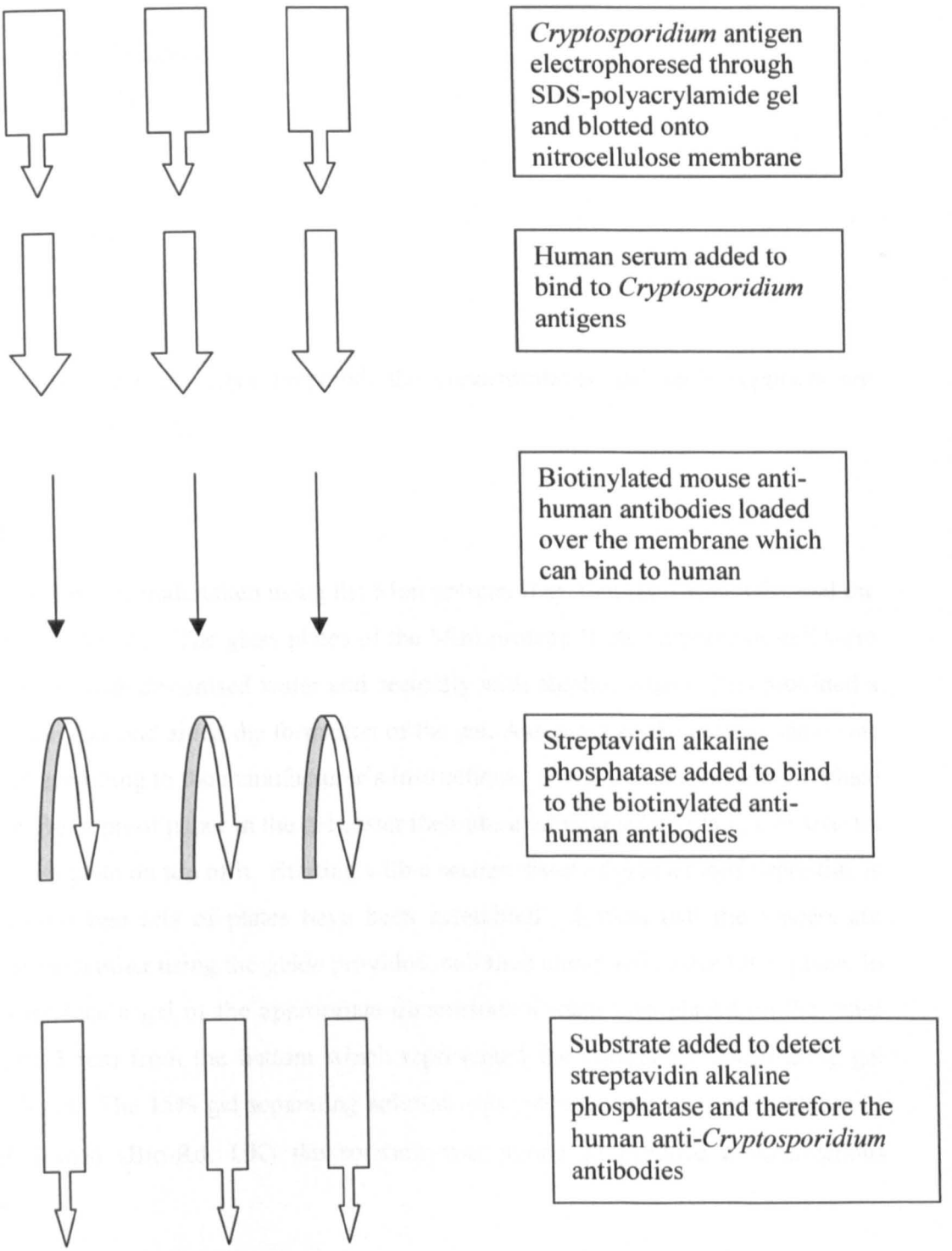
Figure 6.1. Participant from the study population.

6.2.4. The WB method

The presence of anti-*Cryptosporidium* antibodies in the donor serum samples was investigated using immunoblot and immuno-detection techniques. The antigen was electrophoresed on Sodium Dodecyl Sulphate (SDS) polyacrylamide gel (Bio-Rad, UK) to separate the constituent proteins. The SDS is negatively charged; therefore it will coat the proteins and give them all net negative charge which allowed them to migrate toward the positive anode in the presence of an electric field being separated by mass. These proteins were then transferred by semi-dry blotting (Bio-Rad, UK) from the gel onto a nitrocellulose membrane (Schleicher and Schuell, London, UK). An electric current (Bio-Rad, UK) was applied to the gel so that the separated proteins transfer through the gel and onto the membrane in the same pattern as they had separated on the SDS-PAGE. The sites on the membrane which do not contain blotted protein from the gel can be non-specifically blocked so that antibody will not non-specifically bind to the membrane, causing a false positive result. The membrane was then probed with the patient's serum under investigation allowing binding between the

antigen and any corresponding antibodies present in the patient's serum. Antigen/antibody complexes are detected using a multistage approach as illustrated in Figure 6.2. The membrane is incubated with an anti-human antibody that is conjugated with biotin (Zymed Laboratory, INC, San Francisco, USA). This labels any complexes present on the membrane. The membrane is then incubated with streptavidin-alkaline phosphatase (Invitrogen Ltd, Paisley, UK), which binds to any biotin present. The resulting complexes, which are conjugated to the enzyme alkaline phosphatase, are then detected by using the enzyme's substrate (Appendix 15) by a simple colour reaction. The methods were as previously described by (Frost *et al.*, 2000).

Fig 6.2. The Western Blot multi- stages procedure.



6.2.5. Protein electrophoresis

Reagents

15% Separating gel mixture

Temed (Bio-Rad, UK)

Standard antigen

Stacking gel mixture

1X electrode buffer

1X transfer buffer

Alcohol wipes

Details of how reagents were prepared, the concentrations and their suppliers are provided in Appendix 15.

Method

Electrophoresis was undertaken using the Mini protean II system (Bio-Rad, UK) and the gels prepared in pairs. The glass plates of the Mini protean II electrophoresis cell were cleaned firstly with de-ionised water and secondly with alcohol wipes. This provided a clean surface area and aided the formation of the gel. Assembly of the casting stand was performed according to the manufacturer's instructions. Starting from the bottom, place a sheet of greaseproof paper in the gel caster then place an aluminium plate, two spacers and the glass plate on top of it. Starting with a second sheet of greaseproof paper this is repeated until two sets of plates have been assembled. Ensure that the spacers are parallel to each other using the guide provided, and then clamp the caster lid in place. In order to produce a gel of the appropriate dimensions a mark was placed on the outer glass plate 5.5cm from the bottom which represented the correct 15% separating gel solution level. The 15% gel separating solution was prepared and after the addition of 20µl of Temed (Bio-Rd, UK) the solution was mixed to produce a homogenous mixture.

By using a Pasteur pipette the 15% separating gel was injected in between the glass plates of the sandwich clamp assemblies. The gel solution (Appendix 15) was added

until the volume was level with the mark on the glass plate and to produce a smooth gel surface a layer of deionised water was injected on top of the gel. Polymerisation of the gel was completed after 60 minutes at room temperature during which time, 60µl of the antigen was removed from the freezer and prepared along with the biotinylated marker and coloured gel migration dye and the stacking gel was also prepared without the addition of Temed (Appendix15). Once the separating gel had polymerized the casting stand was inverted over the sink to remove the de-ionised water and the corner of a piece of filter paper was carefully inserted in between the glass plates to absorb any excess water. To prevent gel damage touching the separating gel with the filter paper was avoided. A comb (Bio-Rad, UK) with one reference well was then placed between the glass plate and the alumina plate, then the Temed was added to the stacking gel and a Pasteur pipette was used to inject this solution on top of the 15% separating gel and below the comb. The stacking gel fluid level was used to completely fill the remaining space between the glass plates. Polymerisation of the stacking gel was achieved after 10-20 minutes at room temperature. The combs were carefully removed and the two gel forming units fitted into the clamp assembly.

The sandwich clamp assembly was inserted into the buffer chamber, the inner cooling chamber was filled with 1X electrode buffer (Appendix 15) and, after ensuring that there were no leaks, the outer buffer chamber was also filled to a level reaching the lower knob of the sandwich clamp assembly. By using a filter protected tip, 10µl of the marker solution and 45µl of the antigen solution were injected into the smaller reference and the largest wells respectively. The top was placed onto the buffer chamber and the correct coloured wires plugged into the DC current power supply (Bio-Rad, UK). Separation of the protein was achieved by running the gels at 50mA until the dye front reached the bottom of the glass plates and this process lasted 60 min. Turning the power off allowed the safe removal of the electrode on the top of the buffer chamber and consequently the electrode buffer. The sandwich clamp assemblies were removed from the inner cooling core. The glass plates were separated by twisting the spacer bar to access the gel and the gel forming units were

removed from the sandwich clamp assembly. The stacking gel was cut off by using a spare spacer. The gels were transferred prior to blotting, to a plastic dish containing 125ml of 1X Transfer buffer (Appendix 15) and agitated for 5 minutes on a platform rocker (Bibby sterillin, Staffordshire, UK). This was in order to prevent shrinkage of the gel which could cause smearing and also the removal of substances that might inhibit binding of the proteins to the solid phase.

6.2.6. Immunoblotting

Reagents

1X Transfer buffer

PBS/0.3% Tween 20 (VWR International LTD, Leicestershire, UK)

Serum samples

Details of how the reagents were prepared are provided in Appendix 15

Method

Two nitrocellulose membranes (9cm x 7cm)(Schleicher and Schuell, UK) and 4 sheets of extra thick filter paper (10cm x 8cm) (Bio-Rad, UK) were placed in plastic dishes and saturated in 1 X transfer buffer prior to use. A Trans-Blot semi-dry electrophoretic transfer cell (Bio-Rad, UK) was used for the western transfer. For each gel a sheet of extra thick blotting paper was placed on the separating loaded anode platform and piece of nitrocellulose membrane removed from the transfer buffer and placed on top of the blotting paper. The gel was carefully placed on top of the nitrocellulose membrane and a pencil used to note the position of the gel, the corner of the gel and the gel number and the date of the blot. A second sheet of saturated blotting paper was placed on top of the gel and any bubbles or wrinkles in the gel were removed by rolling a pencil over the surface of the gel.

The cathode assembly was replaced along the Trans-Blot SD electrophoretic transfer cell and a DC current of 15v applied to the transfer apparatus for 30 minutes. The apparatus was then disassembled and the gel gently removed off the nitrocellulose membrane and discarded. Each nitrocellulose membrane was placed in separate dishes on platform rocker containing 30ml of PBS/0.3%Tween 20 for 1 hour. The

washed nitrocellulose membranes were placed on top of the Mini-Protean II multiscreen apparatus gaskets (Bio-Rad, UK) and aligned to allow for proper contact of all test lanes on the sample plates. The sample plates were replaced and the screws hand tightened in accordance with the manufacturer's instructions. Then 400µl of human serum (diluted 1:50 in PBS/0.3% Tween 20) (Appendix 15) was injected into each lane and the apparatus were placed on the platform rocker for 1 hour at room temperature and incubated at 4°C overnight. Each blot had a positive control serum.

6.2.7. Immunodetection

Reagents

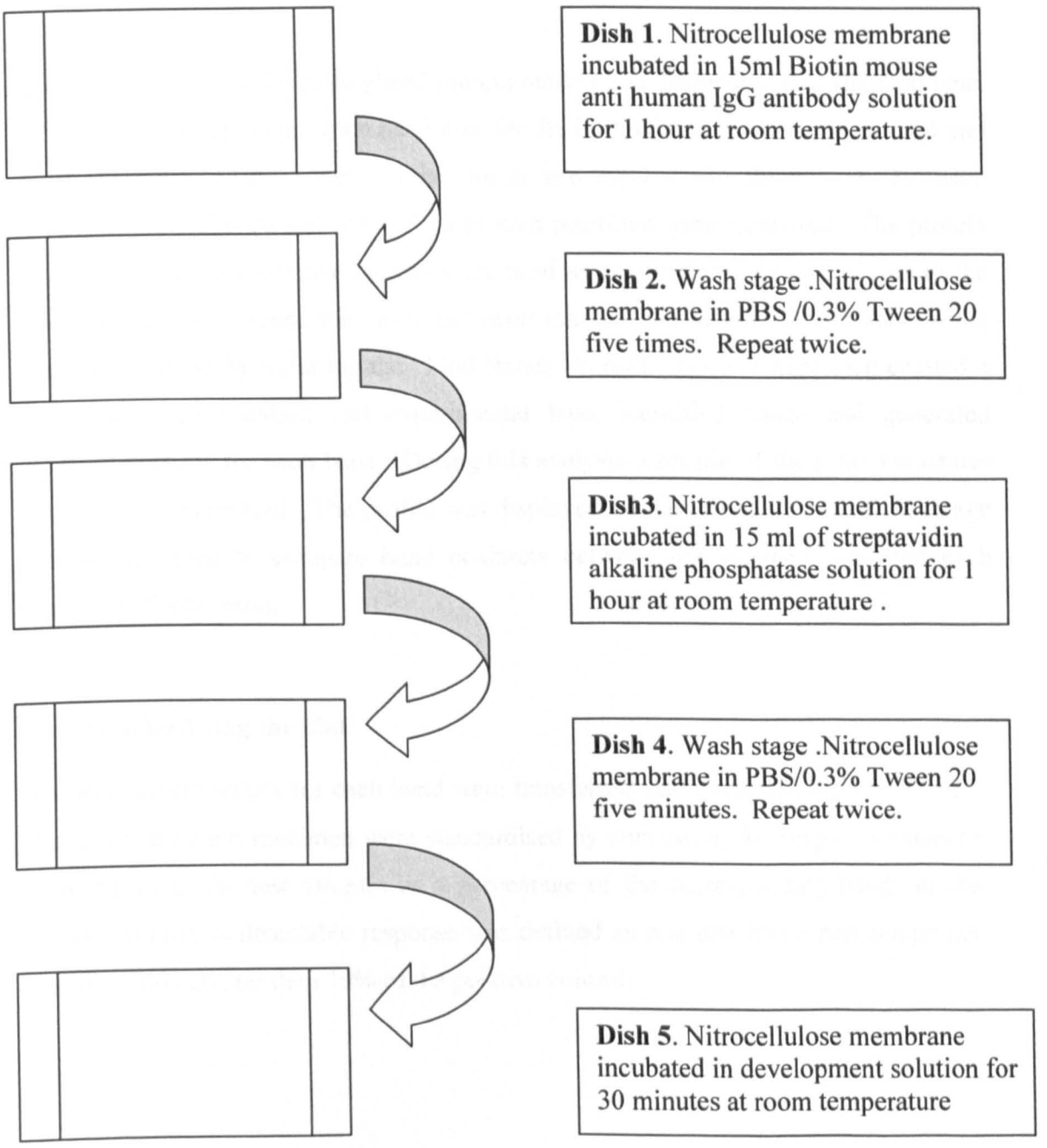
PBS/0.3%Tween 20
Antibody solution
Streptavidin Alkaline Phosphatase
Room temperature developing solution
Distilled water
Details of the reagents are provided in Appendix 15

Method

The serum solutions were removed from each lane in the Mini–Protean II multiscreen apparatus using a vacuum line and replaced with 400µl of PBS/0.3% Tween 20 into each lane of the Mini-Protean multiscreen and it was agitated for 5 min on the platform rocker. The multiscreen apparatus was disassembled and the nitrocellulose membranes were removed and placed on plastic dishes. The dishes were washed for 5 min with 30ml of 1x PBS\0.3% Tween 20 and placed on the platform rocker. This process was repeated twice to ensure sufficient washing of the nitrocellulose membrane.

The nitrocellulose membrane dishes were incubated with 15ml Biotin-conjugated mouse anti-human IgG antibody for 60 min on the platform rocker. The dishes were washed twice with 30 ml of 1xPBS 0.3% Tween 20 for 5 min. A total of 15 ml streptavidin alkaline phosphatase solution was placed into each dish and incubated for 60 min at room temperature. Then the dishes were washed with 30 ml of 1xPBS 0.3% Tween20 twice for 5 min. Finally the dishes were incubated with the room temperature development solution (Appendix 15) for 30 min on the platform rocker. The process was carried out as illustrated in Figure 6.3.

Figure 6. 3. Multistage immunodetection of the antigen/antibody complex.



6.2.8. Blot analysis

Images of the blots were captured using a Kodak Digital Science DC120 Zoom Digital Camera and analyzed using Kodak Digital Science 1D Image analysis software (Anachem, Ltd, Luton Beds, UK). Lane markers were placed to define lanes on the image prior to analysis.

Lane number one was the biotinylated protein marker and designated as a standard lane. By using the pop-up menu, each band size for the biotinylated marker was entered and saved. This allowed every band on the blot to be compared with the marker. For each blot band in the 15KDa and the 27KDa protein positions were identified. The protein band marker and the positive control were used as a guide to help in positioning the bands in each blot. Once the lanes had been marked and labelled, the bands on the image were found by initiating the "Find Bands" button. The software then created a profile for each standard and experimental lane, identified bands and generated statistical analysis for each band. During this analysis a profile of the pixel intensities in each lane was created. The profile was displayed on the left hand side of the image window and used to compare band positions between the standard lane and each experimental lane band.

6.2.9. Standardising the blot

The net intensity values for each band were transferred into a Microsoft Excel ™ file. The results for each specimen were standardised by comparing the response intensity of the bands in the test samples as a percentage of the corresponding bands in the positive control. A detectable response was defined as one that had a percentage net intensity value greater than 10% of the positive control.

6.2.10. Methods of generating results

Two methods were used for generating the results. Firstly, the blots were read by eye and secondly the blots were analysed by using the Kodak Digital Science 1D Image analysis software (Anachem Ltd, UK). Visual analysis of the blots might present problems such as in reproducibility and subjectivity, particularly when the serological response was weak. By capturing an image of the blot using the Kodak Digital camera and analysing it using the software, the intensity of the serological response for each band could be measured objectively. This method would thus eliminate the problem of subjectivity because it allowed a percentage net intensity value to be calculated for each sample and for both antigen groups regardless of whether they were positive, weakly positive or negative. Results were entered and stored in a Microsoft Excel ™ file.

The relationship between the sero-positive individuals and the risk factors included in the questionnaire were tested using an analysis of the variance by using the package, Epi Info 2000 software (CDC, Atlanta, GA). The statistical significance of the different risk factors were tested with Chi-square test, Odds Ratio and 95% confidence interval. Results were considered significant if the P value was < 0.05. The comparison between the visual readings and the computer analysis of the results was assessed by Kappa test.

6.3. RESULTS

6.3.1 Comparison of results using visual interpretation and computer analysis

Both the visual and digital computer analysis methods were used to determine the seroprevalence. The case definition's for comparison of data were each defined as positive results when the participant was sero-positive for the 15-17KDa antigen alone, for the 27KDA antigen alone or for both antigens. Conversely, a negative result was recorded when the participant was sero-negative for both antigens. For the 15-17KDa antigen, 52 (40 %)of the 130 serum samples were scored positive by both methods. However, 5 (3.8 %) were scored positive by eye but negative by digital analysis. A total of 4 samples (3 %) were positive by digital image analysis but negative by eye (Table 6.1).

The 27KDa antigen produces a smaller band of lesser intensity on WB which, makes reading the bands by eye more difficult (Figure 6.3). A total of 70 (53.8 %) of the sera were scored as positive by both methods. However, 9 samples (6.9 %) were scored positive by eye but negative by digital analysis. Six samples (4.6 %) were scored negative by eye but found to be positive by digital image analysis (Table 6.2).

For each of the two antigens, 15-17KDa and 27KDa, there was good agreement with strong kappa correlation values between the computer results and visualizing the blot by eye. However, the computerized method permitted a cut off point to be established and therefore eliminated the problems of subjectivity particularly with weak serological responses. This method should be set as the gold standard for future serological studies using the WB method. All results analysed in the subsequent sections are based on the results drawn from the digital image analysis method.

Table 6. 1. 15-17KDa antigen seropositivity comparing visual and computerized analysis.

15-17KDa antigen (visual analysis)	15-17KDa antigen (computerized analysis)	
	Present	Absent
Present	52 (40%)	5 (3.8%)
Absent	4 (3.1%)	69 (53.1%)
Total	56 (43.1%)	74 (56.9%)

The agreement between the two methods was good as indicated by the Kappa test for agreement (***k*** **test =0.86).**

Table 6. 2. 27KDa antigen seropositivity comparing visual and computerized analysis.

27KDa antigen (Visual analysis)	27KDa antigen by computerized analysis	
	Present	Absent
Present	70 (53.8%)	9 (6.9%)
Absent	6 (4.6 %)	45 (34.6%)
Total	76 (58.5)%	54 (41.6%)

The agreement between the visual and computerized analysis results was good (***k*** **test = 0.75)** .

6.3.2. Prevalence of anti-*Cryptosporidium* antibodies

A total of 130 healthy adults were involved in this study and their serum samples were tested for anti-*Cryptosporidium* antibodies. Of these 67/130 (51.5 %) were females and 63/130(48.5 %) were males. Their mean age was 24 years, and their ages varied from 18 to a maximum of 30 years.

Overall 56/130 (43.1 %) of the participants had antibodies to the 15-17 KDa antigen. Of these 24/56 (42.9%) were males and 32/56 (57.1%) were females. From these 56 sero-positive adults, a total of 45/56(80.4%) were sero-positive to both antigens but 11/56(19. %) of the adults had antibodies to the 15KDa antigen alone. On the other hand, 76/130(58.5%) of the adults had antibodies reactive with the 27KDa antigen with a prevalence of 35/76(46 %) in males and 41/76(54 %) in females. Of these, 31/76(40.8 %) were sero-positive to the 27KDa antigen alone. A total of 43/130 (33.1 %) adults were sero-negative to either antigen.

Thus, by using computerised analysis of WB, a total of 87/130(66.9%) of these adult subjects were sero-positive to one or both of the antigens; thus showing serological evidence for previous infection with *Cryptosporidium* spp. These results are shown in Table 6.3, showing no statistically significant difference in seropositivity between males and females.

Table 6.3. The seroprevalence of anti-*Cryptosporidium* antibodies.

Markers	Male n=63 (%)	Female n=67(%)	χ^2	O.R	95% C.I	*P*
15 KDa	6 (9.5%)	5(7.5%)	0.01	1.31	0.33-5.27	0.9
27KDa	17(27%)	14(20.9%)	0.3	1.40	0.58-3.40	0.5
Both	18(28.6%)	27(40.3%)	1.4	0.59	0.27-1.31	0.2
Neither	22(35%)	21(31.3%)	0.06	1.18	0.53-2.61	0.8

Figure 6.4. The 27KDa narrow bands for selected samples.

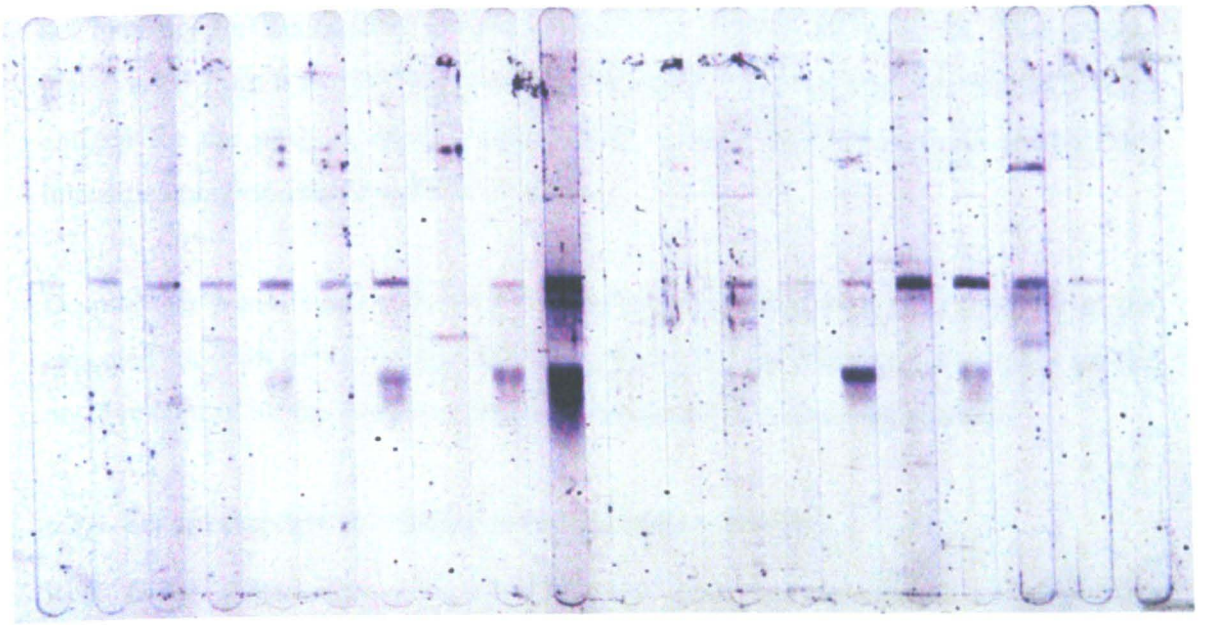
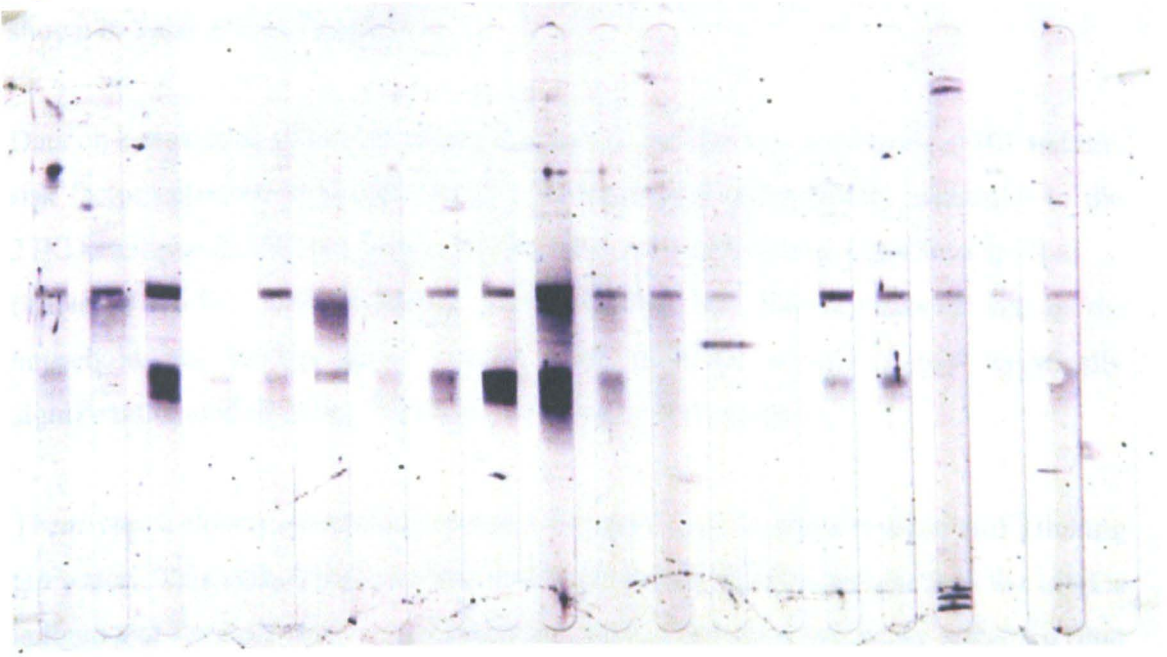


Figure 6.5. The western blot of selected samples.



Lane1 is the colourd marker. Lane 10 is the positive control. Lanes 3,5,6,8,9,11,15,16,19 show samples positive for both markers; Lanes 2,12,13,17,18 show positive samples for the 15KDa antigen marker; Lane 7 represent the positive sample for the 27KDa antigen marker and Lanes 4,14 and 20 are the samples negative for both markers.

6.3.3. Variation among net intensities of the positive control

Band detection was related to the net band intensity. For the 15-17KDa antigen the net intensity for the positive control varied from 2529 to 8270 pixels. The sample blots varied from 0 to 12062.53 pixels in intensity. The net intensity for the 27KDa antigen for the positive control varied from 1294.67 to 8203.26 with sample blot intensities ranging from 0 to 8754.67 pixels.

Despite this blot-to-blot variation by measuring the intensities for each lane at the expected location of the antigen groups and comparing them to the intensity of the positive control on the blot, a cut-off point for positivity can be determined.

6.3.4. Seroprevalence in relation to independent variables

Risk factor information from questionnaire data was compared to serological responses. An increase in the average response ratio with increasing age of subjects for each antigen was observed and this trend was statistically significant for both antigens (P=0.00). The age distribution of the donors whose sera were evaluated is shown in Table 6.4 and Figure 6.6.

Data on exposure to diarrhoea during the past 12 months was collected (n=40) and this risk factor appeared to be associated with increasing seropositivity especially to the 27KDa antigen 23/76(30.3 %) but this trend was not statistically significant (p=0.4) (Tables 6.5-6.7). Neither having young children less than 5 years of age in the household nor having direct contact with domestic animals were statistically significant related to being serpositive to either or both antigens.

There was a strong association between seropositivity to either antigen and drinking tap water. This association was much stronger for the 27KDa antigen than the 15KDa antigen and for each antigen was stronger if the patient drink tap water without a filter than with a filter. There was also link between seropositivity and drinking bottled water (Tables 6.5-6.7).

Table 6. 4. The IgG antibody response to *Cryptosporidium* antigens by age of donor.

Age group	Number (%)	15KDa No (%)	27Kda No (%)	Both antigens No (%)
18-23	34/130 (26.2%)	9/34(26.5%)	21/34(61.7%)	8/34(23.5%)
24-29	53/130(40.8%)	29/53(54.7%)	32/53(60.4%)	15/53(28.3%)
> 30	43/130 (33)	18/43 (41.9)	23/43 (53.5)	23/43 (53.5)

The table shows the distribution of anti-*Cryptosporidium* antibodies to the 15-17KDa, 27KDa and both antigens within each age group. χ^2 for trend =1.34 and P =0.2 for the 15-17KDa, X^2 for trend =0.5 and P=0.4 for the 27KDa and χ^2 for trend =7.3 and **P<0.001** for both antigens.

Figure 6.6 Prevalence of anti- *Cryptosporidium* antibody by age group

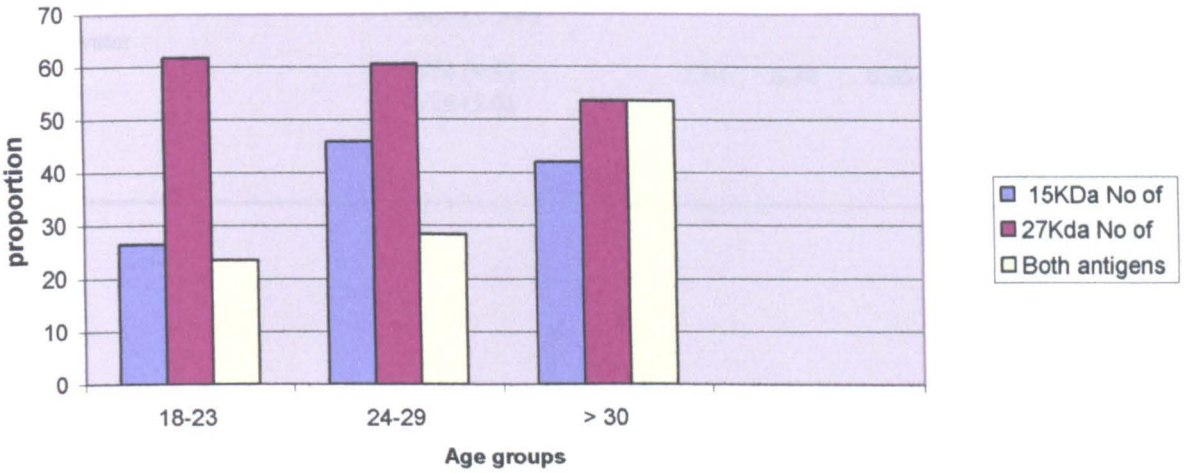


Table 6.5. Independent variables studied in relation to the 15-17KDa antigen serological status of the study population (seropositive n= 56, seronegative n=74).

Variables	Seropositive donors No (%)	X^2	OR	95% CI	P
Diarrhoea (current illness)					
No	2/74 (2.7)	1.36	3.53	0.57-27.5	0.13
Yes	5/56 (9)				
Diarrhoea (during past 12 months)					
No	26/74 (35)	1.10	0.62	0.26-1.42	0.2
Yes	14/56 (25)				
Children in the house					
No	51/74 (69)	0.01	1.13	0.49-2.59	0.7
Yes	40/56 (71.4)				
Domestic animals					
No	14/74 (18.9)	0.01	0.93	0.35-2.49	0.9
Yes	10/56 (17.9)				
Tap water with filter					
No	45/74 (60.8)	4.13	0.48	0.22-1.04	**0.04**
Yes	24/56 (42.9)				
Tap water without filter					
No	22/74 (29.7)	7.55	2.73	1.24-6.02	**0.00**
Yes	30/56 (53.6)				
Bottled water					
No	7/74 (9.5)	1.84	0.34	0.05-1.91	0.1
Yes	2/56 (3.6)				

Table 6.6. Independent variables studied in relation to the 27KDa antigen serological status of the study population (seropositive n= 76, seronegative n=54).

Variable	Seropositive donors No (%)	X^2	OR	95%CI	*P*
Diarrhoea					
No	2/54 (37)	0.10	1.83	0.30-14.2	0.6
Yes	5/76 (6.6)				
Diarrhoea in the last 12 months					
No	17/54 (31.5)				
Yes	23/76 (30.3)	0.65	0.69	0.31-1.54	0.4
Children in the household					
No	40/54 (74)				
Yes	51/76 (67)	0.79	1.49	0.68-3.29	0.3
Domestic animals					
No	11/54 (20.4)				
Yes	13/76 (17)	0.06	0.81	0.30-2.15	0.8
Tap water with filter					
No	35/54 (64.8)				
Yes	34/76 (48.7)	5.11	0.44	0.20-0.96	**0.02**
Tap water without filter					
No	12/54 (22)				
Yes	40/76 (42)	12.16	3.89	1.67-9.22	**0.00**
Bottled water					
No	7/54 (13)				
Yes	2/76 (2.6)	3.75	0.18	0.02-1.02	0.2

Table 6.7. Independent variables studied in relation to the antigen serological status of the study population to both 15-17KDa and 27 KDa antigens (seropositive n= 45, seronegative n=85).

Variable	Seropositive donors No (%)	χ^2	OR	95%CI	*P*
Diarrhoea					
No	2/85 (2.4)	2.88	5.19	0.84-40.6	0.8
Yes	5/45 (11)				
Diarrhoea in the last 12 months					
No	23/85 (27)	1.12	1.64	0.71-3.79	0.2
Yes	17/45 (37.8)				
Children in the household					
No	57/85 (67)	0.65	1.52	0.63-3.73	0.4
Yes	34/45 (75.6)				
Domestic animals					
No	16/85(18.8)	0.01	0.93	0.33-2.60	0.9
Yes	8/45 (17.8)				
Tap water with filter					
No	56/85 (65.9)	46.68	22.58	7.64-70.4	**0.00**
Yes	13/45 (28.9)				
Tap water without filter					
No	22/85 (25.9)	58.30	37.33	11.50-130.6	**0.00**
Yes	30/45 (66.7)				
Bottled water					
No	7/85 (8.2)	0.20	0.52	0.07-2.91	0.6
Yes	2/45 (4.4)				

6.4. DISCUSSION

Infection with *Cryptosporidium* spp causes diarrhoea which may be mild to severe in immunocompetent animals and humans but can be life threatening to immunocompromised individual. Typically, the duration of diarrhoeal illness and ultimate outcome of intestinal cryptosporidiosis depend on the immune status of the patient (Abrahamsen, 1998). Serum and mucosal antibody (Ab) responses coincide with resolution of infection and diarrhoea and may contribute to resistance to or decreased the severity of infection. Serum Ab to *Cryptosporidium* has been detected in both immunocompetent and immunocompromised individuals and in several animal species (Ungar *et al.*, 1990; Ungar *et al.*, 1991; Aguirre *et al.*, 1994). Immunocompetent individuals show typical patterns of IgM and IgG production following infection. The latter may diminish within a few months or persist for a year or more (Campbell & Current, 1983; Ungar & Nash, 1986). Elevated IgA and IgE responses also have been noted (Casemore, 1987). Little is known of the mechanism by which individuals are able to recognize and clear primary infections or develop resistance to subsequent infection (Abrahamsen, 1998).

Recent studies have focused on responses to the 15-17 KDa and 27KDa antigens (Moss *et al.*, 1998; Okhuysen *et al.*, 1998). Antibodies to the 15-17KDa and 27KDa antigens can be considered as very good markers of infection, since the antigens are recognised by IgA, IgG, and IgM serum antibodies in many different species (Reperant *et al.*, 1994). Serological responses to these two antigens appear to be specific for *Cryptosporidium* infection. Infection usually elicits serological responses to these antigens that peak 4-6 weeks after infection. Antibodies to the 15-17 KDa antigen decline to baseline levels observed prior to the infection in 4-6 months after infection while antibodies to the 27KDa antigen remain elevated for 6-12 months (Moss *et al.*, 1998; Priest *et al.*, 1999; Priest *et al.*, 2001; Muller *et al.*, 2001). Antibodies to these antigens have been shown by these workers to be the most consistent in identifying recent infection and therefore for investigating variations in intensity of seropositivity to these antigens may be useful tools to examine different study populations for baseline prevalence of antibody responses.

The finding in the present study that 45/130(34.6 %) of subjects were sero-positive for both antigens is in agreement with previous studies. Previous studies have shown that antibodies to *Cryptosporidium* can be identified in 19 to 60 % of the U.S population depending on age (Chen *et al.*, 1993; Dupont *et al.*, 1995). Sero-prevalences can be even higher in developing nations and in regions with repeated exposure to animals (Ungar & Nash, 1986; Okhuysen *et al.*, 1998). In areas where *Cryptosporidium* is highly endemic the presence of specific cryptosporidial antibodies is extremely common. A study examining the household epidemiology of infection in Urban Brazil demonstrated a seropositivity rate of 94.6 % to *Cryptosporidium* (Heijbel *et al.*, 1987). Specific anti-*Cryptosporidium* antibodies were found in 49.5% of children examined in endemic areas such as three rural communities of Anhui, (Zu *et al.*, 1994)or in more than 60% of the sera obtained from subjects in two low socioeconomic populations in Peru and Venezuela (Ungar *et al.*, 1988). Sero-prevalence studies carried out in adult populations showed the presence of detectable *Cryptosporidium* IgG antibodies in 32 % of US Peace Corps volunteers before they traveled to developing countries (Ungar *et al.*,1989). Another study reported a *Cryptosporidium* IgG sero-prevalence rate of 36% among adults in a farming community in Wisconsin, USA (Lengerich *et al.*, 1993). However, there no similar studies have been reported from the Middle East and Saudi Arabia in particular.

It is highly unlikely that the antibody responses observed in this study could have resulted from cross-reaction with antibodies from a concurrent or previous infection with another organism. This conclusion is because the antibody response to these two antigens is directed against the protein component of the antigens rather than a carbohydrate component. Serum antibody that recognizes surface protein epitopes are most often elicited by infection with the specific pathogen (Priest *et al.*, 2001)

Cryptosporidium differs from other coccidian parasites by recycling sporozoites through the generation of thin walled oocysts. It is therefore not surprising that the host mounts a humoral response to this stage of the life cycle. Antibody cross reactivity between

sporozoite and merozoite antigens may also exist should these stages share antigenic determinants (Mead *et al.*, 1988).

We observed strong positive relationships between drinking water source and the prevalence of serological responses to the two *Cryptosporidium* antigen groups. This study demonstrated a strong association among tap water users and increased sero-positivity to the two *Cryptosporidium* antigens ($p<0.001$). Moreover, increasing serological responses to both antigens 15-17KDa, $p=0.04$ and 27KDa, $p=0.02$ were also even observed among subjects using tap water with a filter. The use of a home water filter did not reduce the prevalence of serological positivity to the two *Cryptosporidium* antigens and the success of bottled water in reducing the infection is expected since drinking water is a risk factor for endemic transmission worldwide. Findings from this study are in agreement with previous studies (Frost *et al.*, 2002; Frost *et al.*, 2003; Hunter *et al.*, 2004) that indicate that water is an important route of transmitting *Cryptosporidium* spp. A two-year follow-up survey following an outbreak of waterborne cryptosporidiosis indicated that the intensity of response to the 27kDa antigen declined to approximately half of its initial value after 2 years (Frost *et al.*, 1998a). What is interesting with regard to that college students survey is that the antibody response to both the 15-17kDa and the 27kDa antigens actually increased after the introduction of a water filtration plant (Frost *et al.*, 2000). This is because the survey was carried out only 5 months post water filtration and may still be a reflection of exposure to *Cryptosporidium* prior to the actual intervention system being implemented.

The observation of a low sero-prevalence among people who regularly drank bottled water raises questions about a causal relationship between drinking water sources and increased risk of infection as indicated previously (Frost *et al.*, 2003; Hunter *et al.*, 2004). These findings suggest that drinking water source characteristics affect the risk of endemic *Cryptosporidium* infection. This study also suggests that populations using

drinking water from major system treated filtration and chlorination and meeting all water quality standards may have an increased risk of water borne *Cryptosporidium* infection.

There is uncertainty over the relative importance of various risk factors for transmission of *Cryptosporidium* infection including the risk from oocyst contaminated municipal drinking water. Among the Saudi donors there was relationship between the occurrence of symptoms consistent with cryptosporidiosis among the past 12 months and seropositivity to either markers. An association was also observed among donors with current diarrhoea and serological responses to both antigens. However, this does not indicate an active infection, as the presence of IgG antibodies does not necessarily correlate with active infection, since oocysts are excreted for a relatively short period (Frost *et al.*, 1998).

The results of this study demonstrated an age-related increase in sero-prevalence of antibodies to *Cryptosporidium* infection. It is possible that mild illness may be associated with strong serological responses. However, previous studies have shown a relationship between seropositivity and exposure to animals (Hunter *et al.*, 2004) or to children (Frost *et al.*, 2001). These relationships were not present in the present study.

It is possible that the high sero-prevalence to *Cryptosporidium* antigens observed in this study indicates an increased levels of protection from clinically significant cryptosporidiosis due to higher level of endemic infection. In a review of a cryptosporidiosis outbreaks, Frost & Craun, (1998b) suggested that previous infections may provide some level of protection against symptomatic infections. Protection from illness but not infection was also observed among individuals experimentally infected who had prior serological responses to the 15-17KDa or 27KDa antigens (Okhuysen *et al.*, 1998; Moss *et al.*, 1998). As a result, asymptomatic infections may predominate in populations with high endemic rates of infection. The findings of the present serological study suggest that endemic *Cryptosporidium* infection has occurred commonly in the population in the Jeddah area, Saudi Arabia. Moreover,

Cryptosporidium disease may be relatively common and primarily asymptomatic or very self-limiting.

Since *Cryptosporidium* infection elicits a serological response in most infected humans (Moss *et al.*, 1998; Frost *et al.*, 1998a); surveys for the presence of this response can be used to estimate the prevalence of prior *Cryptosporidium* infections in populations (Frost *et al.*, 1998a). However, the application of serological methods may not be appropriate in a routine laboratory but they do provide essential information to further understand the epidemiology of *Cryptosporidium*.

CHAPTER SEVEN

GENERAL DISCUSSION AND CONCLUSIONS

Human infection with intestinal parasites is a common health problem in Saudi Arabia (Omar *et al.*, 1991; Milaat & Elassouli, 1995; Al-Braiken *et al.*, 2003). Rapid economic growth in Saudi Arabia and an improved standard of living have been accompanied by importation of expatriate workers with a potential also for importation of disease. This does not mean that the intestinal parasites are not normally found in Saudi Arabia. However, importation of these workers may increase also the importation of the intestinal parasites commonly found in their countries. Female housekeepers arrive in Saudi Arabia mainly from Indonesia, Sri Lanka, Bangladesh, the Philippines, India and Thailand. Many of these workers are infected with various types of parasites and other diseases indigenous to their home countries, which may present potential health hazards. However, with increased awareness and efforts directed towards control of Tropical and Communicable diseases, foreign employees are required to submit a certificate of medical examination before coming to Saudi Arabia. In a study by al-Madani and Mahfouz (1995) a total of 46.5% of surveyed Asian female house keepers in Abha district were infected with one or more parasites. The possibility of spreading such infections throughout the community should be considered in the light of the nature of work of this group being in close contact with different family members.

In the present study intestinal parasites were highly prevalent with 52.2% and 32.3% of the cases of diarrhoeal disease and matched controls having at least one parasite in their stools. This high prevalence is in agreement with previous reports from Saudi Arabia and other countries in the region including Jordan, Gaza, Lebanon, Yemen, Bahrain, Egypt, Iran and Sudan (Amini *et al.*, 1990; Kopecky *et al.*, 1992; Milaat & Elassouli, 1995; Karrar & Rahim, 1995; Mukhtar, 1995; Araj *et al.*, 1996; Yassin *et al.*, 1999; Youssef *et al.*, 2000; Risk & Soliman, 2001; Nimri, 2003). This was reassuring in the validation of our results since only one sample per participant was

examined; nevertheless, the results are very similar to those recorded in previous studies.

G.lamblia and *E.histolytica/dispar* were the two parasites that comprised the largest proportion of the enteric parasites found in the area. The non-diarrhoeal control group had a higher prevalence of helminth infections, however this was not statistically significant, but why this should be is not clear.

The prevalence of the commensal and potentially pathogenic parasites identified in the present study suggested environmental contamination and transmission was high for parasites by the faecal –oral route. Since pathogens as well as commensals are derived from a similar environment, the presence of the latter in patient specimens signifies the potential for acquiring or harbouring parasites causing disease. The presence is generally indicative of existing public health problems. The risk factors for acquisition of intestinal parasites have not been well defined. In general, poor hygiene, and poor levels of socioeconomic development have been thought to be associated with increasing risk of acquisition of intestinal protozoa (Shlim *et al.*, 1999).

The infection rate observed in the present study may well have been higher still if more than one stool specimen had been collected from each child. As in most parasitic infections the shedding of the diagnostic stage of the parasite may be intermittent; even in those patients with massive diarrhoea, two or three faecal specimens may therefore be required.

Epidemiological surveys that use staining and microscopic techniques may underestimate the prevalence of infections, since oocysts can be excreted sporadically or in low numbers (O'Donoghue, 1995) and there is a varying affinity of dyes for the oocysts wall. Alternative methods for antigen detection have been proposed such as ELISA and IF test using monoclonal antibodies (Braz *et al.*, 1996). It is clear from comparison of the detection rates between the diagnostic methods used in the present study that the ELISA test was more sensitive than microscopic identification with the aid of ZN and AP staining techniques and was the only test which identified the

asymptomatic infections. Most but not all of the ELISA positive samples were confirmed by PCR but three samples had no PCR amplicons. It is well known that antibodies directed against *C.parvum* are used in many ELISA kits and these may not react optimally with antigens from other distinct species in the faeces. However, surprisingly the ELISA used in this study identified infections with both *C.meleagridis* and *C.muris*. To the best of our knowledge, this is the first study to report such an occurrence which has implications for the sensitivity and simplicity of using the ELISA technique in epidemiological surveys.

Microscopic detection has the limitation that it is of little use in species differentiation between species of *Cryptosporidium*, since most of the species and genotypes of the parasites are morphologically indistinguishable. This is the case for *C.parvum* and its related species (*C.hominis*, *C.meleagridis*,*C.felis* and *C.canis*) that infect the small intestine 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 cryptosporidiosis is suspected. It is used extensively since clinical diagnosis is usually limited to genus rather than species of *Cryptosporidium* where specific identification is required. For the latter, microscopic identification is unreliable and PCR amplification and genotyping are necessary.

In the present study *Cryptosporidium* infection was found in 9.6% and 0.6% of the diarrhoea cases and controls among Saudi children. Since most laboratories examine stools specifically for *Cryptosporidium* only on physician's request, cryptosporidiosis is generally under-diagnosed in the area. The prevalence of *Cryptosporidium* infection in this study is in agreement with the results of other studies from the Middle East (Stazzone *et al.*,1996; Risk & Soliman, 2001; Igbal *et al.*, 2001; Nimri, 2003). However, the prevalence of the infection is lower than that observed in a study from the same area (Al-Braiken *et al.*, 2003) which reported a prevalence of *Cryptosporidium* infection of 32% and 4.7% in symptomatic and asymptomatic children in 2000. The change in the reported prevalence of cryptosporidiosis during 2001, which is when the collection of samples was performed, might be a consequence of the epidemic of Rift Vally Fever (RVF) in Saudi Arabia in that year.

This reduction in prevalence may have resulted from the public health measures introduced to control the disease epidemic. This may have contributed to the reduction of the prevalence as a result of a decline in contact with animals and a decrease in the size of the animal population as a result of the slaughter policy. However, similar pattern of decrease in prevalence was not apparent for *G.lamblia* and *E.histolytica/dispar* in the present and previous studies. In studies from the UK there were reports of such a decline in the prevalence of cryptosporidiosis as a result of the foot and mouth disease epidemic and this might extend to our observation (Hunter *et al.*, 2003; Smerdon *et al.*, 2003).

In our study 13/24 (54.2%) of the children with cryptosporidiosis were more than 2 years of age. Most epidemiological studies have demonstrated that in developing countries children are those most susceptible and those less than 2 years old usually have the highest prevalence of infection (Reinthaler *et al.*, 1989; Iqbal *et al.*, 1999). This contradiction may be due to relatively high level of exposure to *Cryptosporidium* in the area. The results of a large cohort study among Peruvian children indicated that, although the likelihood of *Cyclospora* attributable diarrhoea decreased significantly following an initial episode of cyclosporiasis, the corresponding trend for *Cryptosporidium* was less consistent and did not achieve statistical significance (Bern *et al.*, 2002). If an initial episode of symptomatic *Cryptosporidium* infection does give some immunity against disease on re-challenge with *Cryptosporidium*, then the prevalence of asymptomatic cryptosporidiosis should increase with age. This is clearly not the case in the present study. It must be remembered, however, that there is a large number of different species and genotypes of *Cryptosporidium* capable of infecting humans with no good evidence, as yet, of cross-protection.

In the second part of this thesis, multilocus PCR-RFLP and sequencing analyses were successfully achieved for *Cryptosporidium* isolates. To the best of my knowledge this is the first study to report the identification of *Cryptosporidium* isolates in Saudi Arabia using microscopy and molecular tools. A variety of PCR/RFLP techniques have been developed to detect and distinguish the *Cryptosporidium* spp that infect humans in particular, *C.parvum* and *C.hominis* (Sturbaum *et al.*, 2001; Spano *et al.*,

1998). In the past, it has been assumed that, in areas where human *Cryptosporidium* infections are endemic, such infections are most likely to be with *C.hominis* (Xiao *et al.*, 2000c; Tiangtip& Jongwutiwes, 2002). However, our results indicate the occurrence of diverse species and genotypes infecting humans. This is in agreement with the published literature on human cryptosporidiosis (Morgan *et al.*, 2000a; Xiao *et al.*, 2001; Tiangtip & Jongwutiwes, 2002). These results showed that at least 17/35 of the isolates from the Jeddah area could have been zoonotic in origin. Of the remaining eighteen samples, four did not yield any PCR products, thirteen were anthroponotic in origin and one patient had a mixed infection with *C.hominis* and *C.parvum*. Most PCR studied have led to the confirmation of *C.parvum* and *C.hominis* as the major causes of cryptosporidiosis in humans. Moreover, recent reports have documented human infections with zoonotic species including with *C.canis* (previously known as the *C.parvum* dog genotype) and *C.felis* (previously known as the cat genotype) (Pedraza-Diaz *et al.*, 2001), *C.meleagridis* (Xiao *et al.*, 2001), *C.muris* (Gatei *et al.*, 2002a), *Cryptosporidium* 'Cervine; genotype (Ong *et al.*, 2002) and *C.parvum* 'pig' genotype (Xiao *et al.*, 2002a) and have been identified infecting both immunocompetent and immunocompromised hosts.

The present data show that in a setting where cryptosporidiosis is a common endemic childhood infection, novel *Cryposporidium* species and novel genotypes of *C.parvum* can infect HIV-uninfected children. In addition to the expected infections with *C.parvum* and *C.hominis,* some children in this study were infected with *C.meleagridis* and *C.muris*. *C.meleagridis* infection was almost as common as that with *C.parvum* bovine genotype in a previous study (Xiao *et al.*, 2001). The results of the present study reveal the diversity of *Cryptosporidium* parasites in one geographic area. Our study also expands the geographic range of suspected *C.muris* infections in humans and suggests that this species may be a global emerging zoonotic pathogen. It is well known that this pathogen may be of particular importance for people living in regions where rodents live in close proximity to humans and sanitation may be minimal. However, *C.muris* may also be more prevalent than currently recognised (Palmer *et al.*, 2003).

In all studies that have identified *C.muris* infection (Guyot *et al.*, 2001; Gatei *et al.*, 2002a; Palmer *et al.*, 2003) including the present study, the parasite was from acute diarrhoeal cases, irrespective the immune status of the patient. However, one study in Indonesia identified *C.muris* infection in two asymptomatic patients (Katsumata *et al.*, 2001).

Initial analysis showed the method of preservation of stool samples is crucial in subsequent DNA recovery and molecular analysis of *Cryptosporidium* spp. Previous studies have used potassium dichromate as preservative for oocysts with storage at 4 °C for subsequent molecular analysis (McLauchlin *et al.*, 2000; Morgan *et al.*, 2000a). However, removal of potassium dichromate prior to DNA amplification is essential (le *et al.*, 1995). Long-term storage of faecal oocysts in formaldehyde has been reported to be possible for subsequent molecular analysis (Zhu *et al.*, 1998). Recently Jongwutiwes and Others (2002) reported that 75% ethanol can be used as an alternative for preservation of *Cryptosporidium* oocysts without significant loss of oocyst morphology and DNA integrity. In the present study we stored a portion of the fresh stool samples at – 80°C without using any preservative.

The 18S or SSU rRNA gene has been used extensively for the identification and characterisation of *Cryptosporidium* parasites (Peng *et al.*, 1997; Xiao *et al.*, 1999a; Morgan *et al.*, 2000a; Fayer *et al.*, 2000; Peng *et al.*, 2001; Ong *et al.*, 2002; Jellison *et al.*, 2002; Gatei *et al.*, 2003;). In our study we analysed the hyper-variable region of this gene and successfully identified four different *Cryptosporidium* species from our study population. By amplification and analysis of this gene fragment, positive identification of both *C.muris* and *C.meleagridis* was possible in addition to detecting the more common *C.parvum* and *C.hominis* species. These results have confirmed the value of analysis of this gene fragment for species and genotypes assignments for *Cryptosporidium.*

Multiple and heterogenous copies of the ribosomal transcription unit are dispersed in the genome of *Cryptosporidium.* It occurs as four copies of the type A gene and one copy of the type B gene per haploid genome (Le Blancq *et al.*, 1997; Xiao *et al.*, 1999b). There is however, only a 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; Sulaiman *et al.*, 2001). This unusual feature of the rRNA locus could make it less suitable for genetic fingerprinting because of the difficulty in distinguishing between intra-genomic heterogeneity and genotypically mixed samples (Widmer *et al.*, 1998c). However, this gene target remains an important tool for the precise identification of *Cryptosporidium* species especially due the wide applicability of a single set of primers for the identification of different species and genotypes.

The COWP gene has been also used for the identification of *Cryptosporidium* spp (Spano *et al.*, 1997b; Xiao *et al.*, 2000b; Chalmers *et al.*, 2002) and it was also selected to amplify cryptosporidial DNA because it has a high degree of polymorphism. This allowed the identification by RFLP analysis of *C.hominis* and *C.parvum* which are present in the majority of human infections (McLauchlin *et al.*, 2000) and of *C.meleagridis* which is has recently been identified as a human pathogen (Xiao *et al.*, 2001). However, polymorphisms within the primers binding sites decreases its sensitivity for *C.muris* amplification and necessitated the use of mixture of oligonucleotide primers (degenerated primers) with some base differences corresponding to the inter-specific sequence variations Amar *et al.*, 2004). This procedure has been used previously and mixture of *Cryptosporidium* spp was detected from shellfish by using this method Gomez-Couso *et al.*, 2004).

No genotypic information was obtained on samples from four subjects. These samples failed to amplify by any of the PCR primers of the 18S rRNA and COWP loci. One sample was confirmed to be positive by microscopy and ELISA, and three samples were positive only by ELISA. One possible reason for not yielding any PCR products is that the oocysts seen were empty (ghosts). Alternatively there may have been an existence inhibitors in the microscopically positive sample, or the ELISA false positive results in these samples that were positive only by ELISA. (the amount of the inhibitors in the microscopically positive sample, or the ELISA false positive was to high to be overcome) Another possible explanation is that the species and genotypes from these samples were genetically diverse from *C.parvum* and *C.hominis*.

Morgan and Others (2000a) indicated that the primers that are specific for *Cryptosporidium* but have been designed on the basis of the *C.parvum* and *C.hominis* genes, frequently do not amplify more genetically diverse genotypes of *C.parvum* or other species.

Previous study has shown that gp15/45/60 encodes two antigenically distinct polypeptides gp45 and gp15 (Strong *et al.*, 2000; Leav *et al.*, 2002). There is no similarity in the deduced amino acid sequences of gp45 and gp15, a finding that is consistent with the finding that antibodies specific for each protein reacted exclusively with the corresponding native or recombinant proteins (Cevallos *et al.*, 2000). Sequence analysis of the 60-KDa glycoprotein (gp /15/45/60), divides *C.hominis* and *C.parvum* into several allelic groups each of which consists of multiple subtypes (Strong *et al.*, 2000; Sulaiman *et al.*, 2001; Glaberman *et al.*, 2002). Results of subtype analysis further support the complexity of human pathogenic *Cryptosporidium*, and five subtype alleles of *C.hominis* and *C.parvum* were found in the present study. Subtype alleles Ia, Ib and Id are characteristic of *C.hominis* and thus were likely of human origin, whereas IIa and Ic are linked to *C.parvum* which could have originated from cattle or humans. The apical and surface localisation of gp45 and gp15 in the invasive stages of the parasite and the finding that they are shed from the surface are consistent with a role in attachment and invasion. Either gp45 or gp15 or both of these glycoproteins may serve as effective targets for specific preventive or therapeutic measures for cryptosporidiosis. (Cevallos *et al.*, 2000), but for example their variability might limits their value as vaccine target candidates.

Extensive genetic diversity within *C.parvum* and *C.hominis* was observed and this can indicate that the gene loci used are under high selection pressure. Previous phylogenetic analysis of *Cryptosporidium* has predominantly examined isolates from natural hosts with zoonotic strains derived from respective animal sources (Xiao *et al.*, 1999; Zhu *et al.*, 2000). In our study, the evolutionary distances in *C.parvum* isolates were very high, however, Alves and Colleagues (2003) reported that *C.parvum* parasites from humans had an even higher genetic diversity than those from animals. Similar profiles of *C.parvum* occurring with high divergences have

been observed in studies using RFLP analysis of the β-tublin gene (Widmer *et al.*, 1998b).

Feng and Others (2002) observed that mixed infections with different genotypes of bovine *C.parvum* produced recombinant progeny when characterised by multilocus genotype analysis comparing alleles inherited from each parental line. This observation represents the first demonstration of sexual recombination in this pathogen. Conversely Tibaryenc and Colleagues (1990) suggested that, for various protozoan species, genetic recombination among genetically distinct strains may be infrequent. While *Cryptosporidium* population structure is almost certainly highly clonal and dominated by these two widespread clonal lineages *C.parvum* and *C.hominis*, occasional interlineage recombination does occur naturally and produces mixed genotype progeny lineages that are viable and infectious (Leav *et al.*, 2002). The clonal hypothesis which states that recombination is rare among natural microbial isolates does not preclude the existence of complete meiosis nor of the occurrence of sexual reproduction but assumes that the opportunity for union of dissimilar genotypes may be limited (Tibaryenc *et al.*, 1990).

Enhanced surveillance and molecular epidemiology have further elucidated the epidemiology of human cryptosporidiosis and shown that regional and seasonal differences exist that may reflect differing exposures and behaviours (McLauchlin *et al.*, 2000). In this study, more infections with *Cryptosporidium* (79.2% of the cases) were observed in the spring during the months of February to April, with only 20.8 % of cases occurring in the winter season between the months of October to January. This seasonal difference may be linked to animal reproduction and husbandry, resulting in a spring increase in human *C.parvum* infections and the winter peak was almost due to *C.hominis*. Analysis of outbreak samples has confirmed that urban transmission is not restricted to *C.hominis* but can occur with *C.parvum*. Although climatic features such as heavy rain and seasonal agricultural practices such as the spreading of excreta as fertiliser are also likely to be of importance (McLauchlin *et al.*, 2000).

Data obtained in this and previous studies indicate the presence of geographic variations in the distribution of cryptosporidiosis infection in human, probably as a reflection of differences in the epidemiology of *Cryptosporidium* infection. In Australia the majority of sporadic cases were identified as being with *C.hominis*, suggesting that infection with anthroponotic parasites played a more important role than infection with zoonotic parasites (Morgan *et al.*, 1995). In contrast, *C.parvum* was responsible for more sporadic human cases than *C.hominis* in the UK (McLauchlin *et al.*, 1999) and France (Guyot *et al.*, 2001).

Our results and those of others illustrate the growing numbers of novel species infecting humans. The identification of a large proportion of infections due to zoonotic species raises questions as to their origin and routes of transmission. In the early 1980s, however, human cryptosporidiosis was generally regarded as a zoonosis (Schultz, 1983), although this was not universally accepted (Casemore & Jackson, 1984).

The host expansion in the *C.parvum* bovine genotype certainly is not only limited to cattle, because this protozoan is now responsible for more infections in humans in Europe than in *C.hominis* (Alves *et al.*, 2001b; Guyot *et al.*, 2001; McLauchlin *et al.*, 2000; Morgan *et al.*, 2000a). However, the latter is still responsible for most human cryptosporidiosis in the rest of the world (Sulaiman *et al.*, 1998; Morgan *et al.*, 2000a; Xiao *et al.*, 2001; Yagita *et al.*, 2001; Leav *et al.*, 2002; Ong *et al.*, 2002; Tiangtip&Jongwutiwes, 2002) perhaps the expansion of *C.parvum* bovine genotype into humans may be due, in part, to the historic development of intensive husbandry practice for various livestock species and the associated high concentrations of young animals at these feeding operation. High concentrations of susceptible hosts such as young calves and lambs can lead to high rates of environmental contamination of as in this case with the bovine genotype *C.parvum*. The clinical outcome of *C.parvum* and *C.hominis* infections in experimentally infected gnotobiotic pigs appears to be determined in part by the *Cryptosporidium* spp used. *C.parvum* strains have a shorter prepatent periods (3-6 vs 8-12 days) and have elicited more disease than *C.hominis* strains (Pereira *et al.*, 2002). The recent finding of *C.meleagridis* in humans which is responsible for as many

Cryptosporidium infection in humans as the *C.parvum* bovine genotype in some areas (Xiao *et al.*, 2001) indicates that host expansion by *C.meleagridis* is also of public health significance. We should also be aware that some other *Cryptospridium* parasites may also have a broad host range and may emerge as 'new' pathogens for humans when socioeconomic and environmental changes favour their transmission (Xiao *et al.*, 2002b).

In the final part of this thesis we were able to assess the sero-prevalence of *Cryptosporidium* in the adult population in the area. In total, 43% and 58.5% of the study population were sero-positive for the 15-17KDa and 27KDa respectively. There is no other such report from Saudi Arabia. The 15-17 KDa and 27 KDa antigens can be considered as very good markers of infection since they are recognised by IgA, IgG and IgM serum antibodies of many different animal species including humans. Humoral immune response cannot be considered as the only, or even the essential effector in recovery or protection from *Cryptosporidium*. Human AIDS sera do contain antibodies specific to the 15-17 and 27 KDa antigens and the parasite is still able to replicate and cause disease. Cellular immunity is required to obtain an efficient protective response (Reperant *et al.*, 1994). Since *Cryptosporidium* infection elicits a serological response in most infected humans (Moss *et al.*, 1998), surveys for the presence of this response can be used to estimate the prevalence of previous *Cryptosporidium* infections in a population (Frost *et al.*, 1998b). Serology enables the detection of exposure to *C.parvum* and prevalence rates recorded in sero-epidemiological studies suggest that cryptosporidial infections are more common than those recorded by coprological methods. Ungar and Others (1988) found that more than 50% of people in Latin American populations with no known history of exposure to infection had specific serum IgG against *Cryptosporidium* (Quilez *et al.*, 1996). A higher prevalence of specific IgG (32 %) has been reported in the North American population but this could represent higher environmental exposure (Braz *et al.*, 1996). This study supports the hypothesis that users of water are at high risk of *Cryptosporidium* infection and the differences between infection rate from epidemiological study and antibody prevalence probably reflect the endemicity of the parasite. These two systematically early recognised

antigens may have a potential for use in prophylaxis /immunotherapy (Frost *et al.*, 2003).

Estimating the risks of infection and understanding risk factors for illness among those infected are critical to understanding the aetiology of this disease and the public health importance of various environmental factors responsible for the transmission of *Cryptosporidium.* In the absence of effective therapeutic agents, although nitazoxanide and azithromycin have shown promise as effective drugs (Doumbo *et al.*, 1997; Smith *et al.*, 1998), control and treatment are dependent upon early and accurate diagnosis and an accurate understanding of the epidemiology and transmission dynamics and highlights the need for preventive measures. The accurate identification of *Cryptosporidium* to species, genotype and sub-genotypes is central to studying transmission patterns and genetic structure and is important for the control of cryptosporidiosis.

The resistance of *Cryptosporidium* oocysts to standard water disinfectants, as well as the low infective dose of viable *Cryptosporidium* oocysts (DuPont *et al.*, 1995) accounts for the risk of waterborne transmission of human cryptosporidiosis and for the serious outbreaks that have been reported (Graczyk *et al.*, 1997). Currently, cryptosporidiosis represents a major public health concern of water utilities in developed nations (Fayer *et al.*, 2000) and reliable detection methods are needed in order to control the presence of the parasite in source and finished water.

The protozoan parasite *Cryptosporidium* has been identified as the cause of foodborne, and day-care centre outbreaks of diarrhoeal disease worldwide (Fayer *et al.*, 1997; Fayer *et al.*, 2000). The persistent of oocysts in the environment allows the environment to be contaminated with more than one species and genotype of *Cryptosporidium.* Multiple genotypes have been detected within single outbreaks (Patel *et al.*, 1998) indicating that even single sources of exposure can contain mixed genotypes (Reed *et al.*, 2002).

Understanding host-parasite co-evolution can also be useful in assessing the public health significance in *Cryptosporidium* parasite from animals. The host-adapted nature of most *Cryptosporidium* parasites indicates that the majority of *Cryptosporidium* parasites probably do not have a high infectivity to humans. Thus, even though *C.muris* and the *C.parvum* 'pig' and 'cervine' genotypes have been found recently in a small numbers of humans (Gatei *et al.*, 2002a; Ong *et al.*, 2002; Tiangtip & Jongwutiwes, 2002; Xiao *et al.*, 2002a) most of whom were immunosuppressed, these parasites are unlikely to be a major human pathogens. On the other hand, the presence of more than one genotype of *Cryptosporidium* parasites in host species such as oppossums, foxes, or cattle reaffirms that humans are not unique in having more than one *Cryptosporidium* species. The host adapted nature of most *Cryptosporidium* spp makes it possible to track the source of *Cryptosporidium* infection in humans and oocysts contamination in the environment. The high prevalence of *C.hominis* in humans indicates that humans are a major source of infection for human cryptosporidiosis. The finding of *C.parvum*, *C.meleagridis*, *C.felis*, *C.canis*, *C.muris*, and *C.parvum* 'pig' genotype and 'cervine' genotypes in humans suggests that farm animals, domestic pets and some wildlife can be potential sources.

For a disease such as cryptosporidiosis to be maintained in a community, there needs to be sufficient density of both susceptible and infected hosts and infectious oocysts available in the environment for transmission. Rational approaches to controlling cryptosporidiosis therefore require an understanding of both the host reservoirs and the routes of infection, together with host susceptibilities and survival of the pathogen in the environment.

Key findings:

♦The prevalence of cryptosporidiosis infection is much higher (9.6%) in the diatrrhoea cases than in the controls (0.6%), Moreover the infection is associated with other symptoms, for example; abdominal cramps, vomiting and fever.

♦Significant differences were found in the overall prevalence of the infection between the winter and spring with high detection rate in the later ($P< 0.00$, $\chi^2 =8.88$, OR= 4.21 and 95% CI= 1.43-13.27).

♦The ELISA test is more sensitive in detecting the infection in faeces than the AP and ZN staining methods.

♦ Contact with other persons with history of diarrhoea in the last 14 days was the only risk factor associated with cryptosporidiosisi infection (P=0.05).

♦This study confirmed the high prevalence of zoonotic infection (*C.parvum* 42.9%, *C.meleagridis* 2.9 % and *C.muris* 2.9%) than the *C.hominis* 37% in the area, this study also found a mixed infection with *C.parvum* and *C.hominis* species in one isolate.

♦Genetic polymorphisms were most extensive in *C.parvum* than in *C.hominis* and the sub-genotypic identification of theses species demonstrated a total of five different allelic groups based on the gp 15/45/60 gene loci.

♦A total of 87/130(66.9%) of the adult subjects involved in this study were sero-positive to one or both of the antigens used.

♦ Source of drinking water with a strong association with drinking tap water was the only factor significantly associated with seropositivity to cryptosporidiosis infection.

Future Work:

♦Research will set up in the future for determining the prevalence and seasonality of the *Cryptosporidium* infection in long period of time and in different part of the country.

♦Identification of the different species, genotypes and sub-genotypes by using other targets (HSP70, TRAP-C1,multilocus Microstallite and ds-RNA)

♦ To validate the detection of anti-*Cryptosporidium* antibodies in saliva

♦Examining the tap water for the presence of *Cryptosporidium* oocysts.

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APPENDIX 1

Parent's information sheet for School children

Subject: Cryptosporidiosis in Jeddah area, SaudArabia

Cryptosporidium is small parasite which mainly infects the human and animal gut' This can cause an illness called cryptosporidiosis which is an important cause of childhood diarrhoea, eecially in developing countries,but some may have no symptoms at all. The diarrhoea usually settles down without special treatment but can make children lose weight and become very dehydrated. If patients also have other illnesses that reduce their resistance to infection, cryptosporidiosis may be much more severe, long-lasting and in rare cases can lead to death.

A study done last year on cryptosporidiosis in the Jeddah area of Saudi Arabia from April-May 2000 suggested that up to 19% of children aged less than 5 years had this infection. The most common symptoms are diarrhoea, weight loss and fever. The organism is spread by the faecal-oral route either by poor hygiene allowing spread from person to person or by contamination of food and water sources with human or animal faeces. The parasite is very resistant to chlorine and this helps it survive in water to spread within communities. Direct contact with animals can also transmit this disease.

The diagnosis usually depends on the microscopic examination of faeces to find the infective and diagnostic stage. There is no specific treatment in normal patients but fluid and (electrolyte) salt replacement are of importance in all cases of diarrhoea.

The aim of this study is to find out how much cryptosporidiosis infection is present in infants and young children (0-6) years and to determine the species of this parasite in the Jeddah area.

Stool samples will be collected to demonstrate the oocysts (the diagnostic stage). A wide plastic container with fitting screwable top and disposable gloves will be despatched to the parents to collect the stool samples. A questionnaire will be sent to the parents to fill in and this will help in collecting some more data to link the parasite with different disease symptoms.

The faecal diagnosis results will be sent to the parents after the microscopic examination. The parents will be informed immediately if their child/children need to visit their own doctor for appropriate treatment.
The teachers will not know the results of individual tests. All the data obtained will be kept confidential and will be deleted as soon as this study has finished. No one else will be told about your child's diagnosis unless you request this.

If you agree for your child to participate in this study please sign the attached consent letter. You are free to refuse to participate and if you do so it will not affect your child's care in any way. You are also free to withdraw your child from the study at any time.

APPENDIX 2

Consent letter for School children

Subject: cryptosporidiosis in Jeddah area, Saudi Arabia

Please fill in the form below:

I confirm that I have read the study information sheet (Appendix 1) and have been able to discuss its contents. I understand that I am free to refuse, or to withdraw my child from the study at any time without any affect on my child's care.

CHILD'S NAME:

SCHOOL NAME:

CLASS NUMBER:

I AGREE TO MY CHILD PARTICIPATING

I DON'T AGREE TO MY CHILD PARTICIPATING

Parent's name: ----------
Signature: ----------

APPENDIX 3

Questionnaire and data for school children

Part 1

Study number:

Date:

School name: Class number:

Family name: First name:

Telephone number:

Parent's educational level: (ring one)

Primary Intermediate Highly educated None

Father's occupation:

Monthly allowance: (ring one)

0-5000 SR/month 5000-10000 SR /month 10000-15000 SR/month More

Part 2

Child's details: (please confirm)

age: months sex: (ring one) M F

height: cm weight kg

Part 3

1-Does your child suffer from any of the following symptoms? (ring one)

Diarrhoea: Yes No

If yes please select the diarrhoea duration (ring only one)

Up to 3 days 4 days - 7 days
8 days -14days More than 14 days

Abdominal cramps: Yes No

Vomiting: Yes No

Loss of appetite: Yes No

Fever: Yes No

Cough: Yes No

2. Has your child had any other diarrhoea in the last 12 months? (ring one)

Yes No

The home environment:

How many people live in your household?

Adults: ----------

Children: (less than 12 years) ----------

Has any one else in the household had diarrhoea in the last 14 days?

Yes No

If yes state how many:

Adults: ----------

Children: (less than 12 years old) --------

Do you have any domestic animals around the home?

Yes No

Has your child have close contact with: (answer yes/no for each):

Cows	Yes	No
Cats	Yes	No
Chickens	Yes	No

Where does your water come from?

APPENDIX 1A

ورقة معلومات الوالدين لأطفال المدارس

الموضوع : كريبتوسبوريديوسيز في منطقة جده – المملكه العربيه السعوديه

الكريبتوسبوريديوسيز هو طفيل صغير يصيب في الدرجه الأولى أحشاء الأنسان والحيوان. هذا يسبب مرض يدعى كريبتوسبوريديوسيز الذي يكون سبب مهم في أسهال الطفوله خاصة في الدول المتطورة ولكن لايوجد لدى البعض أية أعراض. الأسهال عادة يهدأ بدون علاج ولكنه يجعل أطفال يخسرون أوزانهم ويصابون بالجفاف. لو كان المرضى أيضاً يعانون من أمراض أخرى تقلل من مناعتهم للعدوى , الكريبتوسبوريديوسيز تكون حاده أكثر بكثير وتستمر لفترة طويله وفي حالات نادرة تؤدي الى الموت.

طرحت دراسة العام الماضي على الكريبتوسبوريديوسيز في منطقة جدة في المملكه العربيه من أبريل – مايو 2000 أوضحت أن 19% من الأطفال الذين تقل أعمارهم عن 5 سنوات لديه العدوى

أكثر الأعراض الشائعه هي الأسهال و الحمى وانخفاض الوزن.
ينتشر الكريبتوسبوريديوسيز عن طريق البراز اما عن طريق عاده صحيه سيئه تسمح أنتشارالمرض من شخص الى أخر أو عن طريق تلوث مصادر المياه والطعام ببراز الأنسان أو الحيوان. الطفيلي مقاوم جداً للكلور وهذا يساعده على العيش في الماء لينتشر في المجتمعات.

.

التشخيص عادة يعتمد على الأختبارات المجهريه للبراز للبحث عن مرحلة التشخيص والأصابة . لايوجد هناك علاج محدد في المرضى الطبيعين ولكن السوائل وبدائل الأملاح ذات أهميه في جميع حالات الأسهال.

الهدف من هذة الدراسه هو أيجاد عدد العدوى بمرض الكريبتوسبوريديوسيز في الرضع والأطفال الصغار من سن (0 – 6) سنوات ولنقرر فصيلة هذا الطفيلي في منطقة جده .

سوف ترسل نتائج تشخيص الغائط الى الوالدين بعد الأختبارات المجهريه وسوف يبلغ الوالدين حالآ ماأذا كان طفلهم أو أطفالهم بحاجة لزيارة طبيبهم للعلاج المناسب . لن تعرف المعلمات عن النتائج للأختبارات الفرديه. جميع المعلومات التي يحصل عليها سوف تكون سريه وسوف تلغى بعد الأنتهاء من هذه الدراسه. لن يعلم أحد عن تشخيصات طفلك الا اذا أنت طلبت ذلك .

اذا وافقت لطفلك بالمشاركه في هذه الدراسه , الرجاء توقيع خطاب الموافقه الملحق به. لك الحق أن ترفض المشاركه ولو فعلت ذلك لن يؤثر على العنايه بطفلك في أي حال , ولك الحق في أنسحاب طفلك من الدراسه في أي وقت كان

APPENDIX 2A

خطاب موافقه لأطفال المدارس

الموضوع : الكريبتوسبوريديوسيز في منطقة جده – المملكه العربيه السعوديه .

رجاء تعبئة هذا النموذج

أنا أؤكد أني قرأت ورقة معلومات الدراسه (ملحق 1) وأستطعت أن أناقش محتوياتها . أعلم أن لي الحق في رفض أو سحب طفلي من هذه الدراسه في أي وقت بدون أي تأثير على العنايه بطفلي .

أسم الطفل :

أسم المدرسه :

رقم الفصل :

أوافق على مشاركة طفلي.

لاأوافق على مشاركة طفلي

APPENDIX 3A

أستبيان ومعلومات لأطفال المدارس

الجزء الأول

رقم الدراسه:

التاريخ :

أسم المدرسه: رقم الفصل:

أسم العائله: الأسم الأول:

رقم التليفون:

المستوى التعليمي للوالدين (أختار واحده)

أبتدائي متوسط تعليم عالي لاشيء

وظيفة الوالد:

الدخل الشهري (أختار واحده)

(0 -5000 ريال) (5000 – 10000 ريال) (10000 – 15000 ريال)

أكثر

الجزء الثاني

معلومات عن الطفل (الرجاء التأكيد)

العمر : شهراً

الجنس (أختار واحده) ذكر أنثى

الطول سم

الوزن كجم

الجزء الثالث

هل يعاني طفلك من أي من الأعراض التاليه (أختار واحده) 1-

أسهال نعم لا

اذا كانت الأجابه نعم فما هي مدة الأسهال (أختار واحده فقط)

الى 3 أيام 4-7 أيام

8-14 يوم أكثر من 14 يوم

تشنجات بطنيه نعم لا

قيء نعم لا

فقدان الشهيه نعم لا

حمى نعم لا

كحه نعم لا

2-هل عانى طفلك من أي نوع أسهال في الأثنى عشر الشهر الماضيه (أختار واحده)

نعم لا

<u>البيئة المنزليه</u>

1 - كم عدد الأشخاص الذين يعيشون في محيط الأسره؟

البالغين :

الأطفال : (أقل من 12 سنه)

2-هل هناك في المنزل من يعاني من الأسهال في ال14 يوم الأخيره؟

نعم لا

اذا كان الأجابه نعم حدد كم عددهم

البالغين:

الأطفال (أقل من 12 سنه)

- هل تمتلك أي نوع من الحيوانات الأليفه حول المنزل 3

نعم لا

4-هل لدى طفلك أي أتصال بالتالي (أجب بنعم أو لا لكلاً على حده)

الأبقار نعم لا

القطط نعم لا

الدجاج نعم لا

5-من أين تحصل على الماء؟

APPENDIX 4

Parent's information sheet for hospital patients (children)

Subject: cryptosporidiosis in Jeddah area, Saudi Arabia

Cryptosporidium is small parasite which mainly infects the human and animal's gut. This can cause an illness called cryptosporidiosis which is an important cause of childhood diarrhoea especially in developing countries but some may have no symptoms at all. The diarrhoea usually settles down without special treatment but can make children lose weight and become very dehydrated. If patients have other illnesses as well that reduce their resistance to infection, cryptosporidiosis may be much more severe, long-lasting and in rare cases may lead to death.

A study done last year on cryptosporidiosis in the Jeddah area of Saudi Arabia from April-May 2000 suggested that up to 19% of children aged less than 5 years had this infection. The most common symptoms are diarrhoea, weight loss and fever. The organism is spread by the faecal-oral route either by poor hygiene allowing the spread from person to person or by contamination of food and water sources with human or animal faeces. The parasite is very resistant to chlorine and this helps it survive in water to spread within communities. Direct contact with animals can also transmit this disease.

The diagnosis usually depends on the microscopic examination of faeces to find the infective and diagnostic stage. There is no specific treatment in normal patients but fluid and (electrolyte) salt replacement are of importance in all cases of diarrhoea.

The aim of this study is to find out how much cryptosporidiosis infection is present in infants and young children (0-6) years and to determine the species of this parasite in the Jeddah area .

Stool samples to demonstrate the oocysts will be collected. A wide mouth plastic container with fitting, screwable top and disposable gloves will be despatched to the parents to collect the stool samples. A questionnaire will be sent to the parents to fill in and this will help in collecting some more data to link the parasite with different disease symptoms.

The feacal diagnosis results will be sent to the parents after the microscopic examination. The parents will be informed immediately should their child/children need to visit their own doctor for appropriate treatment. All the data obtained will be confidential and will be deleted as soon as this study has finished. No one else will be told about your child's diagnosis unless you request this.

If you agree to your child's participation in this study, please sign the attached consent letter. You are free to refuse to participate and if you do so it will not affect your child's care in any way. You are also free to withdraw your child from the study at any time.

APPENDIX 5

Consent letter for hospital patients (children)

Subject: cryptosporidiosis in Jeddah area, Saudi Arabia

Please fill in the form below:

I confirm that I have read the study information sheet (Appendix 4) and have been able to discuss its contents. I understand that I am free to refuse, or to withdraw my child from the study at any time without any affect of my child's care.

CHILD'S NAME:
HOSPITAL NAME:
FILE NUMBER:

I AGREE TO MY CHILD PARTICIPATING

I DON'T AGREE TO MY CHILD PARTICIPATING

Parent's name: ----------

Signature: ----------

APPENDIX 6

Questionnaire and data for hospital patients

Part 1

Study number:

Date:

Hospital name: Doctor's name:

File number: Date of admission:

Family name: First name:

Telephone number:

Mother's educational level: (ring one)

Primary Intermediate Highly educated None

Father's occupation:

Monthly allowance: (ring one)

0-5000 SR/month 5000-10000 SR /month 10000-15000 SR/month More

Part 2

Child's details: (please confirm)

age: months sex: (ring one) M F

height: cm weight: kg

Part 3

1. Does your child suffer from any of the following symptoms? (ring one)

Diarrhoea Yes No

If yes please select the diarrhoea duration: (ring only one)

Up to 3 days 8days -14days

4 days - 7 days more than 14 days

Abdominal cramps: Yes No

Vomiting: Yes No

Loss of appetite: Yes No

Fever: Yes No

Cough: Yes No

2. Has your child had any other diarrhoea in the last 12 months? (ring one)

Yes No

3. The home environment:

How many people live in your household:

Adults: ----------

Children: (less than 12 years) ----------

Has any one else in the household had diarrhoea in the last 14 days?

Yes No

If yes state how many:

Adults: ----------

Children: (less than 12 years old ---------

Do you have any domestic animals around the home?

Yes No

Has your child have close contact with (answer yes/no for each)

Cows	Yes	No
Cats	Yes	No
Chickens	Yes	No

Where does your water come from ?

APPENDIX 4A

ورقة معلومات الوالدين لمرضى المستشيفات (الأطفال)

الموضوع : كريبتوسبوريديوسيز في منطقة جده – المملكه العربيه السعوديه.

الكريبتوسبوريديوسيز هو طفيل صغير يصيب في الدرجه الأولى أحشاء الأنسان والحيوان. هذا يسبب مرض يدعى كريبتوسبوريديوسيز الذي يكون سبب مهم في أسهال الطفوله خاصة في الدول المتطورة ولكن لايوجد لدى البعض أية أعراض.

الأسهال عادة يهدأ بدون علاج ولكنه يجعل الأطفال يخسرون أوزانهم ويصابون بالجفاف. لو كان المرضى أيضآ يعانون من أمراض أخرى تقلل من مناعتهم للعدوى , الكريبتوسبوريديوسيز تكون حاده أكثر بكثير وتستمر لفترة طويله وفي حالات نادرة تؤدي الى الموت.

طرحت دراسة العام الماضي على الكريبتوسبوريديوسيز في منطقة جدة في المملكه العربيه السعوديه من أبريل – مايو 2000 أوضحت أن 19% من الأطفال الذين تقل أعمارهم عن 5 سنوات لديهم هذة العدوى . أكثر الأعراض الشائعه هي الأسهال و الحمى وانخفاض الوزن.

ينتشر الكريبتوسبوريديوسيز عن طريق البراز اما عن طريق عاده صحيه سيئه تسمح بأنتشار المرض من شخص الى أخر أو عن طريق تلوث مصادر المياه والطعام ببراز الأنسان أو الحيوان. الطفيلي مقاوم جداً للكلور وهذا يساعده على العيش في الماء لينتشر في المجتمعات. الأتصال المباشر مع الحيوان يساعد على نقل هذا المرض.

التشخيص عادة يعتمد على الأختبارات المجهريه للبراز للبحث عن مرحلة التشخيص والأصابة . لايوجد هناك علاج محدد في المرضى الطبيعين ولكن السوائل وبدائل الأملاح ذات أهميه في جميع حالات الأسهال.

الهدف من هذة الدراسه هو أيجاد عدد العدوى بمرض الكريبتوسبوريديوسيز في الرضع والأطفال الصغار من سن (0 – 6) سنوات ولنقرر فصيلة هذا الطفيلي في منطقة جده .

سوف تجمع عينات براز لنظهر بوضوح البويضات (مرحلة التشخيص). سوف يرسل وعاء بلاستيكي عريض للفم مع غطاء متحرك وقفازات تطرح بعد الأستعمال الى الوالدين لجمع عينات البراز

وسوف يرسل أستبيان للوالدين لتعبئته وهذا سوف يساعد على جمع معلومات أكثر لربط هذا الطفيلي مع أعراض المرض الأخرى .

سوف ترسل نتائج تشخيص الغائط الى الوالدين بعد الأختبارات المجهريه وسوف يبلغ الوالدين حالآ ماأذا كان طفلهم أو أطفالهم بحاجة لزيارة طبيبهم للعلاج المناسب . لن تعرف المعلمات عن النتائج للأختبارات الفرديه. جميع المعلومات التي يحصل عليها سوف تكون سريه وسوف تلغى بعد الأنتهاء من هذه الدراسه. لن يعلم أحد عن تشخيصات طفلك الا اذا أنت طلبت ذلك .

اذا وافقت لطفلك بالمشاركه في هذه الدراسه , الرجاء توقيع خطاب الموافقه الملحق به. لك الحق أن ترفض المشاركه ولو فعلت ذلك لن يؤثر على العنايه بطفلك في أي حال , ولك الحق في أنسحاب طفلك من الدراسه في أي وقت كان .

APPENDIX 5A

خطاب موافقة لمرضى المستشفيات (أطفال)

الموضوع : كريبتوسبوريديوسيز في منطقة جدة – المملكه العربيه السعوديه.

رجاء تعبئة هذا النموذج

أنا أؤكد أني قرأت ورقة معلومات الدراسه (ملحق 4) وأستطعت أن أناقش محتوياتها . أعلم أن لي الحق في رفض أو سحب طفلي من هذه الدراسه في أي وقت بدون أي تأثير على العنايه بطفلي .

أسم الطفل :

أسم المستشفى:

رقم الملف:

أوافق على مشاركة طفلي .

لاأوافق على مشاركة طفلي .

أسم الوالد:.

التوقيع:

APPENDIX 6A

أستبيان ومعلومات لمرضى المستشفيات

الجزء الأول

رقم الدراسه:

التاريخ :

أسم المستشفى: أسم الطبيب:

رقم الملف : تاريخ الدخول:

أسم العائله: الأسم الأول:

رقم التليفون:

المستوى التعليمي للأم (أختار واحده)

أبتدائي متوسط تعليم عالي لاشيء

وظيفة الوالد:

الدخل الشهري (أختار واحده)

(0 -5000 ريال) (5000 – 10000 ريال) (10000 – 15000 ريال) أكثر

الجزء الثاني

معلومات عن الطفل (الرجاء التأكيد)

العمر : شهراً

الجنس (أختار واحده) ذكر أنثى

الطول سم

الوزن كجم

الجزء الثالث

هل يعاني طفلك من أي من الأعراض التاليه (أختار واحده) 2-

أسهال نعم لا

اذا كانت الأجابه نعم فما هي مدة الأسهال (أختار واحده فقط)

الى 3 أيام　　4-7 أيام

8-14 يوم　　أكثر من 14 يوم

تشنجات بطنيه	نعم	لا
قيء	نعم	لا
فقدان الشهيه	نعم	لا
حمى	نعم	لا
كحه	نعم	لا

2-هل عانى طفلك من أي نوع أسهال في الأثنى عشر الشهر الماضيه (أختار واحده)

نعم　　لا

<u>البيئة المنزليه</u>

1 - كم عدد الأشخاص الذين يعيشون في محيط الأسره؟

البالغين :

الأطفال : (أقل من 12 سنه)

2-هل هناك في المنزل من يعاني من الأسهال في ال14 يوم الأخيره؟

نعم　　لا

اذا كان الأجابه نعم حدد كم عددهم

البالغين:

الأطفال (أقل من 12 سنه)

- هل تمتلك أي نوع من الحيوانات الأليفه حول المنزل 4

نعم لا

4-هل لدى طفلك أي أتصال بالتالي (أجب بنعم أو لا لكلاً على حده)

الأبقار نعم لا

القطط نعم لا

الدجاج نعم لا

5-من أين تحصل على الماء؟

Appendix 7. Raw data from the hospital questionnaire.

Variable	Cases(n=200)		Controls(n=100)	
	Yes	No	Yes	No
Mother's educational level				
High	110	90	50	50
Intermediate	61	139	39	61
Primary	9	191	2	98
None	20	180	9	91
Monthly allowance				
0-5000 SR	71	129	21	79
5000-10000 SR	105	95	66	34
10000-15000 SR	15	185	10	90
More	9	191	3	97
Symptoms				
1-Diarrhoea				
Up to 14 days	170	30	NA	NA
15-30 days	30	170		
More than 30 days	0	200		
2-Abdominal cramps	161	39	15	85
3-Vomiting	120	80	8	192
4-Fever	79	121	54	46
5-Cough	0	200	0	100
Child with diarrhoea in the last 12 months	59	141	29	71
Contact with other diarrhoea in the last 14 days	49	151	25	75
Contact with domestic animals				
1-Cows				
2-Cats	0	200	0	100
3-Birds	8	192	6	94
4-Chickens	7	193	4	96
	2	198	0	100
Drinking water sources				
1-Tap water	50	150	14	86
2-filttered water	80	120	22	78
3-Botteled water	70	130	64	36

Appendix 8. Raw data from the community questionnaire.

Variable	Cases (n=30)		Controls (n=222)	
	Yes	No	Yes	No
Mother's educational level				
High	21	9	163	59
Intermediate	5	25	28	194
Primary	1	29	17	205
None	3	27	14	208
Monthly allowance				
0-5000 SR	8	22	101	121
5000-10000 SR	11	19	89	133
10000-15000 SR	11	19	24	198
More	0	30	8	214
Symptoms				
1-Diarrhoea				
Up to 14 days	25	5	NA	NA
15-30 days	5	25		
More than 30 days	0	30		
2-Abdominal cramps	20	10	48	174
3-Vomiting	2	28	2	220
4-Fever	4	26	7	215
5-Cough	0	30	0	30
Child with diarrhoea in the last 12 months	9	21	69	153
Contact with other diarrhoea in the last 14 days	4	26	12	210
Contact with domestic animals				
1-Cows	1	29	0	222
2-Cats	6	24	9	213
3-Birds	5	25	3	219
4-Chickens	0	30	1	221
Drinking water sources				
1-Tap water	4	26	86	136
2-filttered water	16	14	80	142
3-Botteled water	10	20	56	66

Appendix 9

LIVERPOOL SCHOOL OF TROPICAL MEDICINE (Affiliated to the University of Liverpool)

Pembroke Place
Liverpool L3 5QA
Telephone 0151708 9393
Fax 01517088733
http//www.liv.ac.uk/lstm/lstm.html

18 May 2001

Dr Faten Al-Braikan C/o Dr
N J Beeching

Dear Dr Al-Braikan

Thank you for resubmitting your research protocol Cryptosporidiosis in the Jeddah area of Saudi Arabia Reference No. 01.26A. This was considered by the Research Ethics Committee on 3 May 2001.

Thank you for your letter of 11 May 2001 with the information requested by the Committee. The protocol now has formal Ethical Approval from the LSTM Research Ethics Committee.

Please remember that this Approval should not be seen as a substitute for Local Ethical Approval from the country/institution where the research is to be carried out and that you have undertaken to seek such approval wherever an appropriate mechanism is in place.

The Research Support Office (RSa) maintains a Database of Local Research Committees in the countries where collaborative work is being carried out. Could you, therefore, feed back to me (via Sharda Mistry in the RSa) as much information as possible on the local CommitteeslReview Bodies that will review (or have reviewed) this protocol. The following details would be much appreciated:

- Name
- Address
- Contact numbers or individuals (tel / fax / e-mail)
- A copy of the appropriate form or some details on the submission mechanism •

Any details you are able to obtain on

a) number on the committee
b) how many lay representatives sit on the committee?

Yours sincerely

Dr Mark Taylor
Chair, Research Ethics Committee

Appendix 10

Sequence analysis for selected isolates from the study based on the 18S rRNA gene.

```
CP80   CTT TAG ACG GTA GGG TAT TGG CCT ACC GTG GCA ATG ACG GGT AAC GGG GAA TTA GGG TTC GAT TCC GGA GAG GGA GCC (78)
CP151  ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... ... ... ... ... ... ... ... ... ... ...
CP115  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP8    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP18   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... C.. ... ... ... ... ... ...
CP129  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP40   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CH41   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ...
CH52   ... ... ... ... ... ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... C.. ... ... ...
CPH68  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ...
CH51   ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... ... ... ... ... ... ... ... ... ... ... ...
CME26  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CMU89  ... ... C.. ... ... ... ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

CP80   TGA GAA ACG GCT ACC ACA TCT AAG GAA GGC AGC AGG CGC GCA AAT TAC CCA ATC CTA ATA CAG GGA GGT AGT GAC AAG (156)
CP151  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP115  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP8    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP18   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP129  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP40   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CH41   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... C..
CH52   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CH68   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CH51   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CME26  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CMU89  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..G .C. ... ... ... ... ...
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CP80   AAA TAA CAA TAC AGG ACT TTT TGG TTT TGT AAT TGG AAT GAG TTA AGT ATA AAC CCC TTT ACA AGT ATC AAT TGG AGG (234)
CP151  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP115  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP8    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ... ... ... ... ... ... ... ...
CP18   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP129  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP40   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CH41   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CH52   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... C.. ... ...
CH68   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CH51   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CME26  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CMU89  ... ... ... ... ... G.C .AA C.. .C. ... ... ... ... ... .G. ... ... ... ... ... ..G ... ... ... ... ...

CP80   CA AGT CTG GTG CCA GCA GCC GCG GT AAT TCC AGC TCC AAT AGC GTA TAT TAA AGT TGT TGC AGT TAA AAA GCT CGT AG(312)
CP151  .. ... ... ... ... ... ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CP115  .. ... ... ... ... ... ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CP8    .. ... ... ... ... ... ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CP18   .. ... ... ... ... ... ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CP129  .. ... ... ... ... ... ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CP40   .. ... ... ... ... ... ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CH41   .. ... ... ... ... ... ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CH52   .. ... ... ... ... ... ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CH68   .C ... ... ... ... ... ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CH51   .C ... ... ... ... ... ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CME26  .. ... ... ... ... ... ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CMU89  .. ... ... ... ... ... ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..

CP80   T TGG ATT TCT GTT AA TAA TTT ATA AT ATT AAT ATT TAT ATA ATA TTA ACA TAA TTC ATA TTA CTA TAT ATT TTA GTA T(406)
CP151  . ... ... ... ... .. ... ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CP115  . ... ... ... ... .. ... ... ... .. ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ... ... ... .
CP8    . ... ... ... ... .. ... ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CP18   . ... ... ... ... .. ... ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CP129  . ... ... ... ... .. ... ... ... .. ... ... ... ... ... .C. ... ... ... ... ... ... ... ... T.. ... ... .
CP40   . ... ... ... ... .. ... ... ... .. ... ... ... ... ... .C. ... ... ... ... ... ... ... ... T.. ... ... .
CH41   . ... ... ... ... .. ... ... ... .. ... ... ... ... ... ... ... .G. ... ... ... ... ... .T. T.. ... ... .
CH52   . ... ... ... ... .. ... ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... ... .T. T.. ... ... .
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CH68  . ... ... ... ... .. ... ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... .T. T.. ... ... .
CH51  . ... ... ... ... .. ... ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... .T. T.. ... ... .
CME26 . ... ... ... ... .. ... ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... A.. TA. ... ... .
CMU89 . ... ... ... ... G. ... .C. ... .. ... ..G G.A ... ..T ... ... T.. AC. .C. T.C C.. T.. ... T.C .A. ... .

CP80  AT AA ATT TTA CTT TGA GAA AAT TAG AGT GCT TAA AGC AGG CAT ATC T TGA ATA CTC CAG CAT GGA ATA ATA TT AAA GA(488)
CP151 .. .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... . ... ... ... ... ... ... ... ... .. ... ..
CP115 .. .. ... ... ... ... ... ... ... ... ... ... G.. ... ... ... . ... ... ... ... ... ... ... ... .. ... ..
CP8   .. .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... . ... ... ... ... ... ... ... ... .. ... ..
CP18  .. .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... . ... ... ... ... ... ... ... ... .. ... ..
CP129 .. .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... . ... ... ... ... ... ... ... ... .. ... ..
CP40  .. .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... . ... ... ... ... ... ... ... ... .. ... ..
CH41  .. .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... . ... ... ... ... ... ... ... ... .. ... ..
CH5   .. .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... . ... ... ... ... ... ... ... ... .. ... ..
CH6   .. .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... . ... ... ... ... ... ... ... ... .. ... ..
CH51  .. .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... . ... ... ... ... ... ... ... ... .. ... ..
CME26 .. .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... . ... ... ... ... ... ... ... ... .. ... ..
CMU89 .G .. .C. ... ... ... ... ... ... ... ... ... ... ... ..A C.. . ... ... ... ... ... ... ... ... A. ..G ..

CP80  T TTT TAT CTT TCT TAT TGG TTC TAA GAT AAG AAT AAT GAT TAA TAG GGA CAG TTG GGG GCT TT GTA TTT AAC GT CAG A(568)
CP151 . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .. ... ... ... .. ... .
CP115 . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .. ... ... ... .. ... .
CP8   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .. ... ... ... .. ... .
CP18  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .. ... ... ... .. ... .
CP129 . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .. ... ... ... .. ... .
CP40  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .. ... ... ... .. ... .
CH41  . ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .. ... ... ... .. ... .
CH52  . ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .. ... ... ... .. ... .
CH68  . ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .. ... ... ... .. ... .
CH51  . ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .. ... ... ... .. ... .
CME26 . ... ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ... ... ... ... ... ... ... .. ... ... ... .. ... .
CMU89 C ... .G. ... ... ... ... ... ..G ..C ..A .G. ... .G. ... ... ... ... ... ... ... ... .C ... ... ... .C ... .
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CP80  GG TGA AAT TCT TAG ATT TGT TAA AGA CAA ACT AAT GCG AAA GCA TTT GCC AAG GAT GTT TTC ATT AAT CAA GAA CGA A(646)
CP151 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CP115 .. ... ... ... ... ... ... ... ... ... ... ... ... ... CG. ... ... .G. ... ... ... ... ... ... ... ... .
CP8   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CP18  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CP129 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CP40  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... C.. ... ... ... ... ... ... .
CH41  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CH52  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CH68  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CH51  .. ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CME26 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CMU89 .. ... ... ... ... ... ... ... ... .G. ... .C. ... ... ... ... ... ... ... ... ... ... ... ... ... .
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Appendix 11

Sequence analysis for selected isolates from the study based on the COWP gene

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CP102 ACT ATG CCT GAA AAA TCA TGT CCC CCA GGA TTC GTT TTT TCT GGA AAA CAA TGT GTT CAA TCA GAC ACA GCT CCT CCT (78)
CP115 ... ... ... ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP18  ... ... ... ... ... ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP40  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ... ...
CP49  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP6   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP8   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CH25  ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ..G ... ... ... ... ... ... ... ... ... ... ... ...
CH41  ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CH52  ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CME26 ... ... ... ... ... ... ... ..T ... ... ... ... ... ... ... ..G ... ... ... ... ... ... ... ... ... ... ...

CP102 AAT CCA GAA TGT CCT CCA GGC ACT ATA CTG GAG AAT GGC ACA TGT AAA TTA ATT CAA CAA ATT GAT ACC GTT TGT CCT(156)
CP115 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP18  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP40  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP49  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP6   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP8   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CH25  ... ... ... ... ... ... ..T ... ... ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CH41  ... ... ... ... ... ... ..T ... ... ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CH52  ... ... ... ... ... ... ..T ... ... ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CME26 ... ... ... ... ... ... ... ... ... ..A ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ...
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CP102 TCT GGT TTT GTT GAA GAA GGA AAT AGA TGT GTT CAA TAT CTC CCT GCA AAT AAA ATC TGT CCT CCT GGA TTC AAT TTG (234)
CP115 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP18  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP40  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP49  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP6   ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ...
CP8   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CH25  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ... ... ...
CH41  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ... ... ...
CH52  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ... ... ...
CME26 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ... ..T ... ...

CP102 TCA GGA CAA CAA TGT ATG GCA CCA GAA TCA GCT GAA TTA GAA TCG ACA TGC CCA CCT AAT TCA ATA TTT GAA AAT GGA  (312)
CP115 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP18  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP40  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP49  ... ... ... ... ... ... ... ... ... ... ... ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP6   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP8   C.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CH25  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CH41  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CH52  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CME26 ... ... ... ... ... ... ... ... ... ..T ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ...

CP102 AAA TGT AAA GTG ATT AAA AAT ATT GAT ATG GTC TGC CCA CCA GGA TAT ACA GAC TCA GGA GAT GAT TGT GTA CTA TAT (390)
CP115 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP18  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP40  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP49  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP6   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP8   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CH25  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ... ... ... ... ...
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CH41  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ... ... ... ... ...
CH52  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ... ... ... ... ...
CME26 ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ...


CP102 GTA GCT CCT GCA AAG GAA TGC CCA CCA AAT TTC ATT TTA CAA GGC CTC CAA TGT ATA CAA ACT AGT TCT GCT CCA ACT (468)
CP115 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ..T ... ... ... ... ... ... ...
CP18  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP40  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP49  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP6   ... A.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CP8   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
CH25  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ..T ... ... ... ... ... ...
CH41  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ..T ... ... ... ... ... ...
CH52  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ..T ... ... ... ... ... ...
CME26 ... ... ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ..G ... ..T ... ... ... ..C ... ... ..A

C102 CAA CCT GTC TGC CCT CCA GGT ACA GT(494)
C115 ... ... ... ... ... ... ... ... ..
C18  ... ... ... ... ... ... ... ... ..
C40  ... ... ... ... ... ... ... ... ..
C49  ... ... ... ... ... ... ... ... ..
C6   ... ... ... ... ... ... ... ... ..
C8   ... ... ... ... ... ... ... ... ..
C25  ... ... ... ... ... ... ... ... ..
C41  ... ... ... ... ... ... ... ... ..
C52  ... ... ... ... ... ... ... ... ..
CME26... ... ..A ... ... A.. ... ... ..
```

APPENDIX 12

Information sheet for adults

Subject: cryptosporidiosis in Jeddah area , Saudi Arabia

Cryptosporidium is a small parasite which mainly infects the human and animal gut. This can causes an illness called cryptosporidiosis which is an important cause of childhood diarrhoea especially in developing countries but some has no symptoms at all. The diarrhoea usually settles down without special treatment but can make children lose weight and become very dehydrated. If patients also have other illnesses that reduce their resistance to infection, cryptosporidiosis may be much more severe, long-lasting and, in rare cases, may lead to death.

A study carried out last year on cryptosporidiosis in the Jeddah area of Saudi Arabia from April-May 2000 suggested that up to 19% of children aged less than 5 years had this infection. The most common symptoms are diarrhoea, weight loss and fever .

The organism is spread by the faecal-oral route either by poor hygiene allowing spread from person to person or by contamination of food and water sources with human or animal faeces. The parasite is very resistant to chlorine and this helps it survive in water to spread within communities. Direct contact with animals can also transmit this disease.

The diagnosis usually depends on the microscopic examination of faeces to find the infective and diagnostic stage. There is no specific treatment in the normal patients but fluid and (electrolyte) salt replacement are of importance in all cases of diarrhoea.

People who have been infected in the past develop special proteins in the blood called antibodies. These can be measured years later in blood and saliva specimens, and this can tell whether a person has had infection in the past. A linked study in looked at the presence of cryptosporidiosis in healthy and ill children. We are not examining diarrhoea specimens in adults but we are trying to find out how many adults have evidence of past infection. A single blood sample will be collected.
5 ml of serum will be taken (an average of a teaspoonful) at the same time as your routine blood test and/or donation, by using the same needle.

A questionnaire will be given to fill in and this will help in collecting some more data to link the parasite with different disease symptoms.

We will not be able to tell you your own results as these are being tested much later in Liverpool, UK. We are studying groups of young men and young women and we are only looking at the total numbers of infected people in each group. We hope that you will be prepared to help in this study to enable us to obtain a wider picture of this infection in the Jeddah area.

All the data obtained will be confidential and will be deleted as soon as this study has finished.

If you agree to participate in this study, please sign the attached consent letter. You are free to refuse to participate. You are also free to withdraw from the study at any time.

APPENDIX 13

Consent letter for adults

Subject: cryptosporidiosis in Jeddah area, Saudi Arabia

Please fill in the form below

I confirm that I have read the study information sheet (Appendix 9) and have been able to discuss its contents. I understand that I am free to refuse, or to withdraw from, the study at any time.

PARTICIPANT NAME

HOSPITAL NAME

FILE NUMBER

I AGREE TO PARTICIPATE

I DON'T AGREE TO PARTICIPATE

APPENDIX 14

Questionnaire and data for adults

Part 1

Study number:

Date:

Hospital name:

Doctor's name:

File number:

Family name: First name:

Age: Sex: (ring one) M F

Telephone number:

Educational level: (ring one)

Primary Intermediate Highly educated None

Occupation:

Monthly allowance: (ring one)

0-5000 SR/month 5000-10000 SR /month 10000-15000 SR/month More

Part 2

1. Do you suffer from any of the following symptoms? (ring one)

Diarrhoea Yes No

If yes please select the diarrhoea duration: (ring only one)

Up to 3 days 8 days – 14 days

4 days - 7 days More than 14 days

Abdominal cramps Yes No

Vomiting	Yes	No
Loss of appetite	Yes	No
Fever	Yes	No
Cough	Yes	No

2. Have you had any other diarrhoea in the last 12 months? (ring one)

Yes No

3. The home environment:

How many people live in your household?

Adults ----------

Children (less than 12 years) ----------

Has any one else in the household had diarrhoea in the last 14 days?

Yes No

If yes state how many:

Adults: ----------

Children: (less than 12 years old) ---------

Do you have any domestic animals around the home?

Yes No

Do you have any close contact with: (answer yes/no for each)

Cows	Yes	No
Cats	Yes	No
Dogs	Yes	No
Chickens	Yes	No

Where does your water come from? (eg. mains, local well, stream)

APPENDIX 12A

ورقة معلومات للبالغين

الموضوع: كريبتوسبوريديوسيز في منطقة جده – المملكه العربيه السعوديه .

الكريبتوسبوريديوسيز هو طفيل صغير يصيب في الدرجه الأولى أحشاء الأنسان والحيوان.

هذا يسبب مرض يدعى كريبتوسبوريديوسيز الذي يكون سبب مهم في أسهال الطفوله خاصة في الدول المتطورة ولكن لايوجد لدى البعض أية أعراض.
الأسهال عادة يهدأ بدون علاج ولكنه يجعل الأطفال يخسرون أوزانهم ويصابون بالجفاف. لو كان المرضى أيضاً يعانون من أمراض أخرى تقلل من مناعتهم للعدوى , الكريبتوسبوريديوسيز تكون حاده أكثر بكثير وتستمر لفترة طويله وفي حالات نادرة تؤدي الى الموت.

طرحت دراسة العام الماضي على الكريبتوسبوريديوسيز في منطقة جدة في المملكه العربيه السعوديه من أبريل – مايو 2000 أوضحت أن 19% من الأطفال الذين تقل أعمارهم عن 5 سنوات لديهم هذة العدوى . أكثر الأعراض الشائعه هي الأسهال و الحمى وانخفاض الوزن.

ينتشر الكريبتوسبوريديوسيز عن طريق البراز اما عن طريق عاده صحيه سيئه تسمح بأنتشار المرض من شخص الى أخر أو عن طريق تلوث مصادر المياه والطعام ببراز الأنسان أو الحيوان. الطفيلي مقاوم جداً للكلور وهذا يساعده على العيش في الماء لينتشر في المجتمعات. الأتصال المباشر مع الحيوان يساعد على نقل هذا المرض. التشخيص عادة يعتمد على الأختبارات المجهريه للبراز للبحث عن مرحلة التشخيص والأصابة . لايوجد هناك علاج محدد في المرضى الطبيعين ولكن السوائل وبدائل الأملاح ذات أهميه في جميع حالات الأسهال.

ينتج الناس المصابين في الماضي بروتينات خاصه في الدم تسمى أجسام مضاده . من الممكن قياسها بعد عدة سنوات في الدم وفي عينة اللعاب وهذه توضح ماذا كان الشخص لديه هذه

الأصابة في الماضي . في دراسة متطورة لوجود الكريبتوسبوريديوسيز في الأطفال المرضى والأصحاء . نحن لانفحص عينات الأسهال في البالغين ولكننا نحاول ايجاد عدد البالغين الذين لديهم الأدلهبأصابه سابقه .

تجمع عينة دم مستقله تؤخذ 5 مل من المصل (بمقدار ملء ملعقة شاي متوسطه) في نفس الوقت الذي يعمل فيه أختبار الدم الروتيني أو التبرع بالدم بأستخدام نفس الأبره .

سوف يعطى أستبيان لتعبئته وهذا يساعد في جمع بيانات أكثر لربط الطفيلي مع أعراض المرض المختلفه .

لن نستطيع أخبارك نتائجك الشخصيه لأننا سوف نقوم بفحصها مؤخراً في ليفربول – المملكه المتحده .

سوف نقوم بعمل دراسات على مجموعات من الشباب والشابات ونحن فقط سوف نرى الأرقام الأجماليه للأشخاص المصابين في كل مجموعه . نتمنى أن تكون مستعداً للمساعده في هذه الدراسه لنستطيع على تحقيق صوره أوسع عن هذه العدوى في منطقة جدة .

أذا وافقت على المشاركه في هذه الدراسه , الرجاء التوقيع على خطاب الموافقه الملحق به . لك الحق أن ترفض المشاركه ولك الحق أن تنسحب من هذه الدراسه في أي وقت

APPENDIX 13A

خطاب موافقه للبالغين

الموضوع : كريبتوسبوريديوسيز في منطقة جدة – المملكه العربيه السعوديه .

الرجاء أكمال المعلومات التاليه .

أنا أؤكد أني قرأت ورقة معلومات الدراسه (ملحق 9) وأستطعت أن أناقش محتوياتها . أنا أعلم أني حر في رفض أو الأنسحاب من هذه الدراسه في أي وقت . أسم المشارك :

أسم المستشفى:

رقم الملف :

أوافق على المشاركه .

لاأوافق على المشاركه .

APPENDIX 14A

أستبيان ومعلومات للبالغين

الجزء الأول

رقم الدراسه:

التاريخ :

أسم المستشفى: أسم الطبيب:

رقم الملف : تاريخ الدخول:

أسم العائله: الأسم الأول:

العمر : الجنس (أختار واحده) ذكر أنثى رقم

التليفون:

المستوى التعليمي (أختار واحده)

أبتدائي متوسط تعليم عالي لاشيء

الوظيفة:

الدخل الشهري (أختار واحده)

(0 -5000 ريال) (5000 – 10000 ريال) (10000 – 15000 ريال) أكثر

الجزء الثاني

1-هل تعاني من أي من الأعراض التاليه (أختار واحده) أسهال نعم

لا

اذا كانت الأجابه نعم فما هي مدة الأسهال (أختار واحده فقط) الى 3 أيام

4-7 أيام

8-14 يوم أكثر من 14 يوم

تشنجات بطنيه نعم لا

قيء نعم لا

فقدان الشهيه نعم لا

حمى نعم لا

كحه نعم لا

2-هل أصبت بأي نوع أخر من الأسهال في الأثنى عشر الشهر الماضيه (أختار واحده)

نعم لا

<u>البيئة المنزليه</u>

1 - كم عدد الأشخاص الذين يعيشون في محيط الأسره؟

البالغين :

الأطفال : (أقل من 12 سنه)

2-هل هناك في المنزل من يعاني من الأسهال في ال14 يوم الأخيره؟

نعم لا

اذا كان الأجابه نعم حدد كم عددهم

البالغين:

الأطفال (أقل من 12 سنه)

3- هل تمتلك أي نوع من الحيوانات الأليفه حول المنزل

نعم لا

4-هل لديك أتصال بالتالي (أجب بنعم أو لا لكلآ على حده)

الأبقار نعم لا

القطط نعم لا

الكلاب نعم لا

الدجاج نعم لا

5-من أين تحصل على الماء؟ (العيون , أبيار محليه , أنهار)

Appendix 15

Reagent preparation for the western blot and their suppliers

1- 1.5 M Tris (PH 8.8) 500ml (BDH, England)

1L bottle was placed on a magnetic stir plate and 350ml deionised water was added with magnetic stir bar to the bottle, while stirring with the magnetic stir bar 90.86g of Tris Ultra Pure was added to the bottle. When the Tris was dissolved, the PH was adjusted to 8.8. The mixture was made up to 500 ml with water and stored at 4 °C.

2 - 0.5M Tris (pH 6.8) 500 ml

1L bottle was placed on a magnetic plate, 350 ml deionised water and magnetic stir bar was added, while stirring with the magnetic bar, 30.29g of Tris Ultra Pure was added to the bottle. When the Tris has been dissolved, the PH was adjusted to 6.8. The mixture was made up to 500ml with water and stored at 4 °C

3 - 0.05 Tris (PH 8.0) 10ml

7ml of deionised water was placed in a 15 ml conical tube and 330µl aliquot of reagent 1. The pH was adjusted to 8 and the mixture were made up to 10ml with water.

4 -1.5 M Tris (PH 7.15) 100ml

A 200 ml bottle was placed on a magnetic stirrer and 75ml deionised water was added. While stirring, 18.17 g of Tris Ultra Pure was added to the bottle. The pH was adjusted to 7.15 and the mixture was made up to 100 ml and stored at 4° C.

5 - PBS (OXOID)

A 1L bottle was placed onto magnetic stir plate and 1L deionised water was added to the bottle. Ten PBS tablets were added to the water, the mixture wasstirred until the tablets were dissolved.

6-PBS/ 0.3 % Tween 10 ml (OXOID)

A 10ml aspirator was placed onto magnetic stir plate and 10L deionised water was added to the aspirator. 100 PBS tablet were added to the aspirator. The mixture was stirred until the tablets were dissolved, then 30 ml Tween 20 was added to the mixture.

7 - 10 % SDS 500 ml (Bio-Rad, UK)

A 1L bottle was placed on a magnetic plate and 450 ml of deionised water and a magnetic stir bar were added to the bottle. While stirring with the magnetic bar, 50g of SDS was added. The mixture was made up to 500ml and stored at room temperature.

8 - Acrylamide/Bis 30 % / 0.8% (Bio-Rad, UK)

9 - Acrylamide 30 % no Bis (Bio-Rad, UK)

7.5ml 40 % acrylamide and 2.5 ml deionised water were added to a universal container. The solution was mixed well and stored at 4 °C

10 - Temed (Bio-Rad, UK)

11 - 10 % Ammonium Persulphate 300 µl (VWR International LTD, UK)

30mg of Ammonium persulphate was added to a micro centrifuge tube. A 300 µl aliquot of deionised water was added to the tube .

12 - 5X Electrode buffer 1L

A 1L bottle was placed on a magnetic plate and 750 ml of deionised water and a magnetic stir bar were added to the bottle. While stirring with the magnetic bar ,15 g of Tris Ultra Pure (BDH), 70.2g of Glycine (VWR International LTD, UK), and 5g of SDS (Bio-Rad, UK) were added to the bottle. The mixture was made up with water to 1L and stored at 4 ° C .

13 - 1 X Electrode buffer 1L

200ml of 5 X electrode buffer (reagent 12) was placed into a graduated cylinder, then the solution was made up with water to 1L and stored at 4 ° C .

14 - 5 X Transfer buffer 1L

A 1L bottle was placed on a magnetic plate and 750ml of deionised water and a magnetic bar were added to the bottle. While stirring, 15g of Tris Ultra Pure (BDH) and 70.2 g of Glycine (Bio-Rad, UK) were added to the bottle. The mixture was made up to 1L with water and stored at 4 ° C.

15 - Methanol (VWR International LTD, UK)

16 - Transfer buffer-250ml

A 50ml of 5X transfer buffer (reagent 14) and 50 ml of methanol were added to a 250 ml graduated cylinder. The mixture was made up to 250ml with water.

17 - 0.5 M Magnesium Chloride 500ml (Sigma, UK)

A 1L bottle was placed on a magnetic plate and 350ml of deionised water was added to the bottle. While stirring, 23.8g of Magnesium Chloride (Sigma, UK) were added to the bottle. The mixture was made up to 500 ml and stored at 4 ° C.

18 - 15% Separating Gel (4gel-volume)

The following reagents was placed into a 50 ml flask; 7.5 ml of reagent 1, 300 µl of reagent 7, 7.5 ml of reagent 8 ,7.5 ml of reagent 9, 8.7 ml deionised water, 300 µl of reagent 11. The contents were mixed and then 30µl of reagent 10 was added.

19 - Stacking gel (4 gel - volumes)

The following reagents were placed into a 50ml flask; 5ml of reagent 2; 200µl of reagent 7; 3ml of reagent 8; 200µl of reagent 11 and 11.6 ml deionised water. These contents were mixed and then 24µl of reagent 10 was added.

20 - Sample (Laemli) buffer (Sigma , UK)

21 - Antigen preparation

The antigens in ultra micro centrifuge tubes were removed from the freezer. A total of 150µl of reagent 20, 4µl of reagent 3 and 60µl of antigen were placed into a 1.5ml ultra micro centrifuge tube. This tube was placed in a 65 °C dry heating block for 15 minutes.

22 - Antibody preparation 30ml

Into a flask, 30ml of reagent 6 and 60µl aliquot of Biotin-Mouse Anti Human IgG (Zymed Labs Inc, USA) were placed. These contents were mixed well.

23 - Streptavidin-Alkaline Phosphatase 30 Ml

Into a 50 ml flask the following reagents were added; 30 ml of reagent 6 and a 30µl aliquot of Streptavidin-Alkaline Phosphatase (Invitogen, UK), these contents were mixed well.

24 - Stock Nitro Blue Tetrazolium solution (NBT) 393 ml (Sima, UK)

In a 500ml bottle the following reagents were placed and stored at 4 ° C ; 13ml of reagent 4, 2.4ml of reagent 17, 377.6ml of water and 100mg of NBT

25 - Dimethylformamide (Sigma, UK)

26 – Stock 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) 4ml
In a container, 4ml of reagent 25 and 50mg of BCIP were placed, mixed and stored at 4 ° C.

27 - Developing solution (2 blots)
In a 50ml beaker the following reagents were placed immediately prior to use; 39.3ml of reagent 24 and 400µl of reagent 26.

28 - Dilution buffer 50 ml
In a bottle, 0.5g of skimmed milk powder and 50ml of 1x PBS/ 0.3 % Tween were added and mixed well.

29 - Patients serum samples
The serum samples were removed from the –20 ° C freezer and allowed to completely thaw. These serum tubes were vertoxed and an 8µl aliquot of each sample has been placed into a 0.5 ml centrifuge tube and a 400µl aliquot of reagent 28 were placed into the tube.

30 - Blocking buffer 100 ml
In a bottle, 5g of skimmed milk powder and 100ml of 1x PBS/ 0.3% Tween were placed and mixed.

31 - Pefabloc solution

In a container, 5ml of reagent 5 and Img of Pefabloc (Sigma, UK) were added and mixed.

32 - Pepstatin A 1ml (Sigma, UK)

In a container 1mg of Pepstatin A and 1ml of reagent 6 were placed and mixed.

33 - Leupeptin 1 ml (Sigma, UK)

In a container, 1mg of Leupeptin and 1ml deionised water were placed and mixed.

34 - NaOH 0.5 M

In a plastic container 0.2g NaOH and 10ml deionised water have been placed and mixed.

35 - EDTA solution (100 µg/ µl) 10 ml(Sigma, UK)

In a container, 1000mg of EDTA and 10 ml of reagent 34 were placed and mixed.

36 - Sample buffer 12 ml

In a universal container 10ml of reagent 20, 10µl of reagent 23, 10µl of reagent 33 and 2ml of reagent 35 were placed and mixed.

37 - Mrker (Bio-Rad, UK)

In a 0.5 ml tube, 20µl of reagent 20, 1µl of Biotinylated SDS-page Low Range Protein Marker, and 2µl of pre stained SDS-page Low Range Marker were placed and incubated at 100°C for 5 minutes. The tube was allowed to cool at room temperature before use.

Kingdom of Saudi Arabia
Ministry of Health
The Directorate of Health Affairs - Jeddah
Administration of Continuous
Training & Education

المملكة العربية السعودية
وزارة الصحة
المديرية العامة للشئون الصحية بمحافظة جدة
إدارة التدريب والتعليم المستمر

الرقم : ٨٥٣/٥٢٦/٤/٢ No. التاريخ ١٤٢٢/٩/١١ Date المشفوعات Attached

سعادة / مدير مستشفى الولاده والأطفال بجده **الموقر**

السلام عليكم ورحمة الله وبركاته

بالإشاره إلى خطاب سعادة عميد كلية الطب والعلوم الطبيه رقم ٣٣٣٢/ط/٤٢٢ وتاريخ ١٤٢٢/٧/٢٢هـ المتضمن طلب الموافقه على السماح لطالبة الدراسات العليا بالكلية أ / فاتن بنت عبدالله البريكان بجمع عينات (لبراز ولعاب) من بعض المرضى من الأطفال ذوي الست سنوات ، وذلك لإستخدمها في بحث الدكتوراه الخاصه بالمذكوره .

عليـــه نفيـــدكم بالموافقه على إجراء هذا البحث .

نأمل منكم الإطلاع وتسهيل مهمتها .

وتقبلوا أطيب تحياتي ، ، ،

مدير الشئون الصحيه بمحافظة جده

د/ سمير بن عبدالله لنجاوي

ك/ح

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P33

ANTIBIOTIC USE IN COMMUNITY-ACQUIRED PNEUMONIA

A. N. J. Malik and G. E. Packe, Respiratory Medicine, Newham General Hospital, London

The British Thoracic Society (BTS) has published detailed guidelines on the management of community-acquired pneumonia.[1] To determine if these were being followed in our hospital a retrospective analysis was conducted of patients admitted between January to December 2001.

94 patients met the study criteria. 62% were male. Median (range) age was 59 years (20–97). 87% received intravenous (IV) antibiotics, compared to a predicted 30–50% quoted in the BTS guidelines. Only 33% of patients received initial antibiotics that were from the recommended combinations. 45% were receiving oral cephalosporins after stopping IV antibiotics, although Amoxicillin or Co-amoxiclav are specifically recommended. 88% of those with no recorded severity factor had IV antibiotics. The recommended time to the first antibiotic dose is 2 hours. 21% of direct GP referrals and 13% of referrals from the A&E department met this target.

BTS guidelines were not followed in any of the areas examined. Inappropriate use of parenteral antibiotics resulted from the variable interpretation of basic severity factors for pneumonia.

1. BTS Pneumonia guidelines committee. BTS Guidelines for the Management of Community Acquired Pneumonia in Adults. Thorax 2001; 56: Supp IV.

P34 *

PREVOTELLA MELANINOGENICA AS A CAUSE OF SEPTIC SHOCK IN A SPLENECTOMISED PATIENT

K. Forbes, A. Cadwgan, G. Franklin and R. Laing, The Infection Unit, Aberdeen Royal Infirmary, AB25 2ZN, Scotland

A fifty-seven year old man was admitted to the Regional Infection Unit with a one-day history of rigors and general malaise. He had a splenectomy during childhood as a part of treatment for a portal vein thrombosis. On arrival he was shocked, cyanosed and drowsy, with a marked metabolic acidosis and renal impairment. He failed to respond to fluid resuscitation and was transferred within an hour to the ITU. His ITU stay was 10 days during which time he received inotropic and ventilatory support, haemofiltration and latterly haemodialysis. Blood cultures on admission grew *Prevotella melaninogenica* (a gram negative anaerobe similar to *Fusobacterium necrophorum*) resistant to penicillin. Initial antimicrobial therapy was with cefotaxime and gentamicin. Metronidazole was added on receipt of a provisional blood culture report. During his admission to the ITU he developed digital ischaemia of his hands and feet. Following discharge from the ITU he remained in hospital for a further 11 days. His renal function recovered to normal but amputation of three fingers on his left hand was necessary. Organisms other than the 'traditional' capsulated pathogens need to be considered in splenectomised patients resenting with sepsis.

P35

BCG AS A CAUSE OF CHEST ABSCESS SECONDARY TO BLADDER INFECTION?

G. Mowat[1], J. G. Magee[1], J. Heatherington[1] and P. Unsworth[2], [1]Newcastle Public Health Laboratory and [2]Tameside General Hospital

Bacillus Calmette Guérin (BCG) has long been used to stimulate immunity against tuberculosis. It has also been used to stimulate anti-tumour immunity. Most therapeutic uses of BCG have been abandoned but intra-vesical instillation remains effective for transitional cell carcinoma of the bladder. In 80% of patients treatment results in long-term elimination of the tumour. However, there is a possibility of renal abscesses and local overgrowth by BCG leading to prolonged urinary excretion of organism and the risk of dissemination and systemic illness. We report here the case of an 80-year old patient who, after 18 months instillation therapy, developed a chest wall abscess which yielded a growth of an *Mycobaterium tuberculosis* complex organism. Given the age of the patient and the elapsed time from bladder instillation, the isolate was potentially coincidental and may have indicated recrudescence of classical tuberculosis. Phenotypic characterisation was inconclusive but the isolate was finally identified as the vaccine strain by PCR confirmation of the RD1 deletion.

P36

PREVALENCE AND SEASONALITY OF *CRYPTOSPORIDIUM* INFECTIONS IN JEDDAH, SAUDI ARABIA

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Background: Few studies have been published on cryptosporidiosis in the Middle East although prevalence rates of 19% and 6.7% have been described in children in Gaza and Jordan, respectively.

Aims: To describe the seasonality of cryptosporidiosis and to define paediatric age groups most affected.

Methods: (1) Retrospective review of laboratory records for 1998–2001 inclusive from a large public hospital (KAAH, Jeddah). (2) Prospective examination of faecal specimens collected from 253 children, aged from 0 to 5 years, March–May 2000. Subjects were recruited from six pre-schools and four different paediatric clinics in local hospitals.

Results: Cryptosporidiosis is much more common in the spring season, and the most frequent age group identified with infection is from 12 to 24 months. 20/63 (31.7%) children with symptoms were diagnosed with *Cryptosporidium* spp, compared to 9/190 (4.7%) asymptomatic children. There was no difference in the prevalences of other pathogens.

Conclusion: Cryptosporidiosis is common in young children with diarrhoea in the Jeddah area, particularly during spring.

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Detection of *Cryptosporidium* amongst diarrhoeic and asymptomatic children in Jeddah, Saudi Arabia

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Between the March and May of 2000, stool specimens were collected from children aged <5 in six pre-school crèches and clinics in the Jeddah area of Saudi Arabia. Sixty-three (25%) of the children had diarrhoeal disease but the other 190 were asymptomatic. When the stool samples were stained and examined for the oocysts of *Cryptosporidium* spp. and other enteric protozoa, 20 (32%) of the symptomatic children but only nine (4.7%) of the asymptomatic were found to be excreting *Cryptosporidium* oocysts. Similarly, *Cyclospora cayetanensis* was found, always as a co-infection with *Cryptosporidium* sp., in seven (11%) of the children with diarrhoeal disease but only eight (4.2%) of the asymptomatics. One asymptomatic child was found to be co-infected with *Isospora belli* and *Cryptosporidium*. *Giardia intestinalis* and cysts of *Entamoeba histolytica/dispar* were detected in 18 (29%) and nine (14%) of the children with diarrhoeal disease and in eight (4.2%) and 13 (6.8%) of the asymptomatic children, respectively. This appears to be the first report of *Cy. cayetanensis* from Saudi Arabia and the highest prevalence of *Cryptosporidium* infection ever described.

Coccidian parasites are being increasingly recognised as important causes of diarrhoea in children and immuno-compromised patients world-wide (Cegielski *et al.*, 1999). Species of *Cryptosporidium* are ubiquitous in their distribution, and various genotypes of *Cr. parvum* and anthropophilic and zoonotic species such as *Cr. meleagridis*, *Cr. muris* and *Cr. felis* have all been implicated in human illness (Morgan *et al.*, 1998; Morgan-Ryan *et al.*, 2002; Tiangtip and Jongwutiwes, 2002; Gatei *et al.*, 2003).

In temperate climates, cryptosporidiosis accounts for only 1%–4% of the cases of childhood diarrhoea and is usually self-limiting (although it can produce devastating diarrhoea in HIV-positive individuals who have fewer than 200 $CD4^+$ lymphocytes/μl). In tropical environments, however, cryptosporidiosis is much more prevalent, accounts for 4%–20% of the cases of childhood diarrhoea, and is associated with high morbidity (Mathan *et al.*, 1985; Sallon *et al.*, 1991; Hart, 1999; Iqbal *et al.*, 1999) and mortality in children (Molbak *et al.*, 1993).

There appear to be few published reports of studies on cryptosporidiosis in the Middle East. Between 6.7% and 19% of the symptomatic children investigated in Gaza (Sallon *et al.*, 1991, 1994) and Jordan (Nimri and Hijazi, 1994) were found to be infected with *Cryptosporidium*. Cross-sectional studies in Kuwait (Daoud *et al.*, 1990; Iqbal *et al.*, 2001), Egypt (Khashba *et al.*, 1989; Mikhail *et al.*, 1989; Stazzone *et al.*, 1996) and the Sudan (Robinson *et al.*, 1986) and among Bedouin children in Israel (Dagan *et al.*, 1991)

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revealed infection prevalences of 1.6%–10%. In Saudi Arabia, Khan *et al.* (1988) found *Cryptosporidium* oocysts in two (1%) of 209, routinely processed, faecal samples from children with diarrhoea attending clinics at the Maternity and Children's Hospital in Dammam. In a similar study at King Abdul Aziz University Hospital in Riyadh, *Cryptosporidium* oocysts were also found in stool samples from 1% (two of 174) of the children investigated (Bolbol, 1992). In none of these reports from the Middle East was infection with other enteropathic protozoa, such as *Isospora belli* and *Cyclospora cayatensis*, reported, although a case of isosporiasis in an HIV-infected patient has been described in Dakar, Senegal (Dieng *et al.*, 1994). Isosporiasis causes a similar spectrum of illness to cryptosporidiosis in children and immunodeficient patients (Curry and Smith, 1998). The geographical distribution of *Cyclospora* infection appears more patchy than those of *Cryptosporidium* or *Isopora*, and links between *Cyclospora* and severe illness in immuno-deficiency have not been definitively established (Cegielski *et al.*, 1999).

In Saudi Arabia, diarrhoeal disease (DD) is an important cause of morbidity in children but the contribution made to it by coccidian parasites is unknown. The primary aim of the present, pilot study, based in the Jeddah area, was to determine and compare the prevalences of *Cryptosporidium* in young children with and without DD.

SUBJECTS AND METHODS

The subjects were children, aged <5 years, from the Jeddah area of Saudi Arabia who, between the March and May of 2000, attended six (randomly selected) crèches or, because of diarrhoea, presented at four paediatric clinics. The purpose of the study was explained to the principal of each crèche and to the parents or guardians of all the children. A child was only investigated if one of his or her parents or guardians gave his or her informed consent. In the crèches, teachers and nursery attendants were asked to record the age and sex of each child and the presence or absence of DD. The attendants were provided with disposable gloves and were instructed in the safe collection of a single faecal sample from each child. Similar data and stool samples were collected, routinely, in the clinics.

Before it could be examined, each stool sample was stored cool, in 10% formalin, for <24 h. Each sample was strained through a fine (0.6-mm-pore) sieve, mixed with 3 ml of ether or ethyl acetate for 1 min and then centrifuged at 1000 × *g* for 1 min. The sediment left after the supernatant fatty plug, debris and liquid had been discarded was mixed well and then used to prepare two smears on clean slides. One smear was examined wet, unfixed and unstained. The other was allowed to air dry before being fixed in methanol (3 min), stained with unheated carbol fuchsin (15 min), washed with tap water and then counterstained with 0.4% methylene blue (30 s). Each smear was checked carefully for parasites, under a light microscope, by a microscopist in Jeddah who was unaware whether the sample came from a symptomatic or asymptomatic child. For quality control, all of the parasite-positive smears and a proportion of the negative were rechecked by an experienced parasitologist in Liverpool.

The prevalence data were collected and analysed using version 6.03 of the Epi Info software package (Centers for Disease Control and Prevention, Atlanta, GA).

RESULTS

All six crèches approached agreed to participate in the study and together provided stool samples from 190 children, all of whom were considered asymptomatic by their nursery attendants. A further 63 stool samples, all from children with DD, were obtained from the four paediatric clinics. There were similar numbers of male and female subjects, both overall (55.7% female)

and in each age-group considered in the data analysis (see below). Forty-three of the children investigated were aged < 1 year. At least one species of protozoan parasite was detected in 120 (47.4%) of the 253 stool samples (Table 1). Of the 120 samples positive for protozoan parasites, 61 came from girls and 59 from boys. *Cryptosporidium* oocysts were detected in the stools of 20 (32%) of the children with DD but only nine (4.7%) of the asymptomatic children ($P < 0.001$; Table 2). Similarly, *Giardia intestinalis* was found much more frequently among the diarrhoeic children than among the asymptomatic (29% *v.* 4.2%; $P < 0.001$). *Entamoeba histolytica* and *E. dispar* could not be distinguished with the techniques available. Infection with *E. histolytica/dispar*, however, was also more frequently detected in the subjects with diarrhoea than those without (14% *v.* 6.8%; $P = 0.03$; Table 2). The individual role of *Cyclospora cayetanensis* could not be assessed as this parasite was only detected as a co-infection with *Cryptosporidium*. However, the co-infection appeared significantly associated with DD, being seen in 11% of the diarrhoeic children but only 4.2% of the asymptomatic ($P = 0.05$). Only one child was found to be excreting *I. belli* and he was asymptomatic though co-infected with *Cryptosporidium*. Among the children aged < 2 years or 3–5 years, those with DD were significantly more likely to be excreting *Cryptosporidium* occysts that those who appeared asymptomatic (Table 3).

TABLE 1. *Parasite distribution among the 141 girls and 112 boys investigated*

Parasite	No. and (%) of: Males infected	Females infected
Cryptosporidium alone	17 (15)	12 (9)
Cyclospora cayetanensis and *Cryptosporidium*	4 (4)	11 (8)
Isospora belli and *Cryptosporidium*	1 (1)	0 (0)
Giardia intestinalis	11 (10)	15 (11)
Entamoeba histolytica/dispar	13 (12)	9 (6)
Other*	15 (13)	11 (8)

**Ascaris lumbricoides*, hookworm and/or *Enterobius vermicularis*.

DISCUSSION

It is estimated that DD is responsible for more than 2 million deaths in children under 5 years of age, most of those deaths being in developing countries (Hart *et al.*, 2002). Rotavirus is the commonest cause of acute watery diarrhoea in children throughout the world (Hart and Cunliffe, 1999). *Cryptosporidium* spp., however, are usually the third or fourth commonest cause of DD, and the prevalence of *Cryptosporidium* infection is much higher in developing countries than elsewhere (Fayer and Ungar, 1986; Morgan *et al.*, 1998; Hart, 1999). Previous hospital-based studies in Saudi Arabia have revealed a low prevalence (1%) of *Cryptosporidium* infection among children with diarrhoea

TABLE 2. *Parasite distribution among the 63 symptomatic and 190 asymptomatic children*

Parasite	No. and (%) infected: Symptomatic children	Asymptomatic children	*P*
Cryptosporidium alone	20 (32)	9 (4.7)	<0.001
Giardia intestinalis	18 (29)	8 (4.2)	<0.001
Entamoeba histolytica/dispar	9 (14)	13 (6.8)	0.03
Isospora belli and *Cryptosporidium*	0 (0)	1 (0.5)	–
Cyclospora cayetanensis and *Cryptosporidium*	7 (11)	8 (4.2)	0.05
Other*	11 (17)	15 (7.8)	0.03

**Ascaris lumbricoides*, hookworm and/or *Enterobius vermicularis*.

TABLE 3. *Detection of* Cryptosporidium *by age-group*

	No. and (%) of symptomatic children:		No. and (%) of asymptomatic children:		
Age-group (months)	Investigated	Infected	Investigated	Infected	*P*
0–12	5	2 (40)	38	0 (0)	0.01
13–24	14	5 (36)	43	2 (5)	0.00
25–36	10	3 (30)	23	4 (17)	0.35
37–48	14	7 (50)	35	5 (14)	0.01
49–60	20	10 (50)	51	7 (14)	0.00

(Khan *et al.*, 1988; Bolbol, 1992). In the present study, the prevalence of mono-infection with *Cryptosporidium* among the children with diarrhoea (who were presenting to paediatric out-patient clinics) was much higher (32%) and appears to be the highest ever reported (Hart, 1999). This indicates that there is a particularly high level of exposure to *Cryptosporidium* in this setting, an observation further emphasised by the discovery that 4.7% of the asymptomatic children were excreting cryptosporidial oocysts. In Vellore, India, Mathan *et al.* (1985) also found oocyst excretion to be quite frequent (9.8%) among asymptomatic children. The results of a large cohort study among Peruvian children indicated that, although the likelihood of *Cyclospora*-attributable diarrhoea decreased significantly following an initial episode of cyclosporiasis, the corresponding trend for *Cryptosporidium* was less consistent and did not achieve statistical significance (Bern *et al.*, 2002). If an initial episode of symptomatic *Cryptosporidium* infection does give some immunity against disease on rechallenge with *Cryptosporidium*, then the prevelance of asymptomatic cryptosporidiosis should increase with age. This is clearly not the case in the present study population (Table 3). It must be remembered, however, that there is a large number of different species and genotypes of *Cryptosporidium* capable of infecting humans (Gatei *et al.*, 2003), with no good evidence, as yet, of cross-protection.

Cyclospora cayetanensis can cause DD in both immuno-competent and immuno-compromised individuals. Infection with this parasite has been detected in a number of different geographical regions, including North, Central and South America, the Indian sub-continent and South-east Asia (Herwaldt, 2000). In Europe, infection with *Cy. cayetanensis* is most often associated with travellers returning from visits overseas (Crowley *et al.*, 1996; Jelinek *et al.*, 1997). Although Osman *et al.* (1999) observed *Cy. cayetanensis* in Egypt, albeit at low prevalence (1.3%), there appear to be no previous reports of this species in Saudi Arabia. In the present study, interestingly, all of the 15 children found to be infected with *Cy. cayetanensis* were co-infected with *Cryptosporidium*. Co-infection with these two coccidia was significantly associated with DD. Although there are superficial similarities between *Cy. cayetanensis* and *Cr. parvum*, there are also considerable differences (Herwaldt, 2000; Bern *et al.*, 2002). In Peru, human infections with either parasite are more common during the warm season than during the cooler part of the year (Bern *et al.*, 2002). Oocysts of *Cryptosporidium*, however, are fully infective when excreted whereas those of *Cy. cayetanensis* are unsporulated on excretion and require several days outside the host to sporulate (Herwaldt, 2000). Co-infection with *Cy. parvum* and *Cr. cayetanensis* has been reported before, but only in an adult with AIDS (Scaglia *et al.*, 1994).

Only one case of *I. belli* infection was detected in the present study and that in an asymptomatic child. *Isospora* is known to be a rare cause of childhood diarrhoea. In India, for example, Mirdha *et al.* (2002) only

detected seven cases of symptomatic isosporiosis among 4112 children with DD. No *I. belli* infections were observed in the diarrhoeic Egyptian children studied by Osman *et al.* (1999).

In conclusion, the present results reveal a very high prevalence (32%) of *Cryptosporidium* mono-infection and a high prevalence (11%) of *Cyclospora/Cryptosporidium* co-infection among children with DD attending paediatric outpatient clinics in Jeddah, Saudi Arabia. It is clear that *Cryptosporidium* infection is an important cause of DD but one that does not always necessitate hospital management.

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