CRYPTOSPORIDIOSIS IN TWO REGIONS OF SAUDI ARABIA

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

By

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DEDICATION

I dedicate this thesis to my parents, my wife and our children and to my brothers, for their love and support in all the time. Also to my dead supervisor Prof. Anthony Hart for his kindness, guidance and advice.

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I wish to express my deepest gratitude to my supervisors, (the late) Professor Anthony Hart and Dr Nicholas Beeching for their kindness, guidance and advice.

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ABSTRACT

This is the first study on human and animal infections with *Cryptosporidium* spp. in Gizan and Maddina regions of Saudi Arabia. Between March 2005 and September 2006, stool samples from 1641 people (predominantly children) were screened for *Cryptosporidium spp.* These included 454 patients with diarrhoea and 62 controls in Gizan and 1125 patients from Maddina. In addition, 279 animal samples were examined from Gizan, including cows, goats and sheep.

The human samples from Gizan were initially screened by microscopy using modified Ziehl-Neelsen (ZN) and Safranin Methylene Blue staining techniques, while the animal samples were only screened using a modified ZN staining technique. The control samples were all negative by both methods. ZN identified *Cryptosporidium* oocysts in 21 (4.6%) samples and SM-B identified oocysts in 26 (5.7%) out of 454 human samples from Gizan. ZN only identified *Cryptosporidium* oocysts in one sheep sample. Stools from Maddina were not routinely examined by microscopy unless positive by other means.

All 1920 faecal samples (human or animal) were evaluated using an Enzyme Immunoassay (EIA), which detected *Cryptosporidium* Specific Antigen in 104 (5.4 %) of the samples (103 human and one animal). Comparison of the detection rates between the diagnostic methods, i.e. microscopy and EIA, using a subgroup of 454 samples from Gizan, showed 100% agreement in specificity between the two methods, but EIA was more sensitive (5.7% vs 9.9%).

In both Gizan and Maddina regions, cryptosporidiosis was more common in children less than two years of age and the maximum prevalences were observed during the cooler months.

All 104 samples (46 from Gizan, 58 from Maddina) containing *Cryptosporidium* oocysts were initially amplified by 18S rRNA-based PCR. The 101 (97.1%) samples positive for *Cryptosporidium* were further genotyped by an 18S rRNA-based PCR-Restriction Fragment Length Polymorphism (RFLP) technique. The 104 samples were also amplified using PCR for GP60 and HSP70 genes, for multilocus sub-genotyping.

Among the 101 samples which were positive by 18S rRNA PCR, 79 (78.2 %) isolates were subsequently classified by RFLP as *C. parvum*, 13 (12.9 %) as *C. hominis* and one (1%) was *C. felis* (from a sheep in the Gizan area). 8 human samples from the Gizan area contained mixed infections with *C. parvum* and *C. hominis*, as determined using RFLP of 18S rRNA gene.

95 (91.3%) and 88 (84.6%) of the 104 samples containing *Cryptosporidium* oocysts were successfully amplified for sub-genotype studies, using PCR for the GP60 and HSP70 genes respectively. Sequence analysis of study isolates of these two loci in general confirmed the species identification obtained by the18S rRNA gene PCR- RFLP analysis. In total, allele groups IIa, IIc and IId of *C. parvum* and allele groups Ib and Ie of *C. hominis* were obtained by GP60 sequencing. HSP70 sequence analysis was less successful and did not add much to discrimination.

Cryptosporidiosis was common in children, and screening of stools by EIA was twice as sensitive as light microscopy after special staining. The predominant species in humans was zoonotic *C. parvum*, with some *C. parvum* subtypes associated with anthroponotic spread also identified by multilocus sequencing. PCR amplification of both GP60 and HSP70 genes was successful, but GP60 was the most useful additional tool, showing extensive genetic heterogeneity in *Cryptosporidium* spp. at the subtype level. Although this may correlate with origin of the infection, studies in local animals were unsuccessful in confirming possible animal sources.

ABBREVIATIONS

AFS	Acid Fast Stain
AP	Auramine Phenol
BFM	Bright Field Microscope
BLAST	Blast Local Alignment Search Tool
Bp	Base Pairs
BSA	Bovine Serum Albumin
°C	Degree Centigrade
Cb	Community based
CD4+T	Cluster of Differentiation 4 T Lymphocytes
CDC	Centers for Disease Control and Prevention
CHEF	Contour-Clamped Homogeneous Electrophoresis Field
CI	Confidence Interval
COWP	*Cryptosporidium* Oocyst Wall Protein
CSA	*Cryptosporidium* Specific Antigen
dhfr-ts	Dihydrofolate Reductase-Thymidylate Synthase gene
DFA	Direct Immunofluorescent Assay
DMM &GUM	Department of Medical Microbiology and Genito-Urinary Medicine
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
DWM	Direct Wet Mount
EDTA	Ethylenediamine Tetraacetate
EIA/ELISA	Enzyme Immunoassay/Enzyme Linked Immunosorbent Assay
E&I	Eosin & Iodine stain
F	Female
FE	Formol-Ether
FIGE	Field Inversion Gel Electrophoresis
FM	Fluorescent Microscope
GC	Guanine-Cytosine
GP60	Glycoprotein Sporozoite Antigen 60 Kilo Dalton
Hb	Hospital based

HAART	Highly Active Antiretroviral Therapy
HIV/AIDS	Human Immune-Deficient Virus/Acquired Immune – Deficiency Syndrome
HPLC	High Performance Liquid Chromatography
HSP70	Heat Shock Protein 70 Kilo Dalton
I	Inpatient
ICZN	International Code of Zoological Nomenclature
ID50	Infectious dose 50%
IF	Immuno-Flourescent
IPTG	Isopropyl-ß-D-Galactopyranoside
ITS-1	Internal Transcribed Spacer1
ITS-2	Internal Transcribed Spacer2
kDa	Kilo Dalton
LB	Luria Broth
M	Male
MCK	Modified Cold Kinyoun
MF	Methiolate Formaldehyde
Ml	Millilitre
ML	Maximum Likelihood
MP	Maximum Parsimony
NCBI	National Center for Biotechnology Information
NJ	Neighbour-Joining
Nr	Not reported
O	Outpatient
ORH	Oral Rehydration Therapy
P	Probability value
PCR	Polymerase Chain Reaction
PE	Perkin Elmer
pGEM-T	Vector system (Promega)
Poly(T)	Polythreonine
RAPD	Randomly amplified polymorphic DNA

RFLP	Restriction Fragment Length Polymorphism
Rpm	Round per minute
rRNA	Ribosomal Ribonucleic Acid
RNR	Ribonuclease reductase
S	Sedimentation
SAF	Acetic acid and Formaldehyde
SDB	Specimen Dilution Buffer
SFC	Sugar Flotation Concentration
SMB	Safranin-methylene blue
SSCP	Single-Strand Confirmation Polymorphism
*Ssp*1	*Sphaerotilus* derived endonuclease
SSU rRNA	Small Subunit Ribosomal Ribonucleic Acid
TBE	Tris Borate EDTA
TMB	Tetramethyl-Benzadine
TRAP-C1	Thrombospondin Related Adhesion Protein1 *Cryptosporidium* gene
TRAP-C2	Thrombospondin Related Adhesion Protein2 *Cryptosporidium* gene
TSSP	Trichrome Stool Smear Preparation
UK	United Kingdom
µl	Microlitre
USA	United States of America
*Vsp*1	*Arthrobacter* derived endonuclease
w/v	Weight by Volume
ZN	Ziehl-Neelsen

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1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Cryptosporidium is a coccidian protozoan parasite of the intestinal tract that causes severe, potentially fatal watery diarrhoea in immunocompromised patients and self-limiting but often prolonged diarrhoeal disease in immunocompetent individuals (Soave & Armstrong, 1986; Framm & Soave, 1997). It can also infect other animal species and thus may be zoonotic (Angus, 1983; Tzipori, 1983).The infective form of *Cryptosporidium* is the thick-walled oocyst which is excreted in large numbers in faeces during acute infection. The oocyst is resistant to desiccation and most disinfectants (Hart, 1999). Volunteer studies have demonstrated that the infective dose can be as low as one oocyst (Hart, 1999) and thus infection is easily spread person-to-person, either directly or indirectly.

Cryptosporidiosis is a significant cause of diarrhoeal disease in both developing and industrialized nations (Sulaiman *et al.*, 2005) but several epidemiological studies have demonstrated that *Cryptosporidium* is more prevalent in developing countries (5% to >10%), than in developed countries(<1% to 3%) (Reinthaler *et al.*, 1989; Current & Garcia, 1991; Iqbal *et al.*, 1999).

In temperate climates, cryptosporidiosis accounts for only 1% to 4% of the cases of childhood diarrhoea and is usually self-limiting (Hart, 1999). It can, however, produce devastating diarrhoea in HIV-infected individuals who have fewer than 200 CD 4+ lymphocytes/ µl of blood (Al-Braiken *et al.*, 2003). In tropical environments, cryptosporidiosis is much more prevalent accounting for 4% to 20 % of the cases of childhood diarrhoea, especially in children less than two years of age(Lima & Guerrant, 1992; Hart, 1999). It is associated with high morbidity (Mathan *et al.*, 1985; Sallon *et al.*, 1994; Hart & Cunliffe, 1999; Iqbal *et al.*, 1999) and this probably reflects a poorer nutritional status in children in developing countries (Macfarlane & Horner-Bryce, 1987). Transmission of *Cryptosporidium* spp. via contaminated drinking water, outdoor and indoor recreational waters and municipal water is well documented (Rush *et al.*, 1987; Smith *et al.*, 1988).

Recent molecular epidemiological studies of cryptosporidiosis have helped us to gain better understanding of the transmission of cryptosporidiosis in humans and the public health significance of *Cryptosporidium* spp. in animals and the environment.

1.2 History

Cryptosporidium was first described in the laboratory mouse by Tyzzer in 1907 and named *C. muris* (Tyzzer, 1907). In 1912, he described *C. parvum* in the large intestines of mice (Tyzzer, 1912). In 1955, Slavin described the parasite as a potential cause of diarrhoea in turkeys. Cryptosporidiosis in calves was subsequently recognized in the 1970s (Panciera, 1971).

Although the first case of human infection was described in 1976 (Meisel *et al.*, 1976; Nime *et al.*, 1976) it was not until *Cryptosporidium* infections were reported as a cause of death in AIDS patients in the 1980s that the protozoan parasite became accepted as a significant zoonotic pathogen warranting scientific research (Current *et al.*, 1983). *Cryptosporidium* sparked great public health interest after the large human waterborne outbreak in Milwaukee in 1993 (MacKenzie *et al.*, 1995) and rapidly became recognized as one of the most serious and difficult to control waterborne pathogens described.

Different species of *Cryptosporidium* have been named in the literature on the basis of biological and genetic studies. In recent years molecular characterizations of *Cryptosporidium* have helped to clarify the confusion in *Cryptosporidium* taxonomy and validate the existence of multiple species in each vertebrate class. As a result several new species of *Cryptosporidium* have been named (Xiao & Ryan, 2004).

1.3. Life cycle

The life cycle of most *Cryptosporidium* species is completed within the gastrointestinal tract (primarily small intestine and colon) of the host with developmental stages being associated with the luminal surface of the mucosal epithelial cells. It is direct and monoxenous and it follows the patterns described for other enteric coccidia which include a merogonic cycle with two generations of meronts, a gametogonic cycle with macrogametes, microgametes and zygotes and a sporogonic cycle (de Graaf *et al.*, 1999).

The infectious form is the oocyst and following ingestion by the host, sporozoites are released from it and infect host gut epithelial cells, (lung epithelial cells can also be infected by sporozoites). The sporozoites then differentiate into trophozoites, which in turn differentiate into type I meronts. Merozoites produced by type I meronts are released from the host cell and can infect other host cells. These merozoites can then either initiate asexual reproduction by differentiating into trophozoites, or initiate the sexual cycle by differentiating into type II meronts. Oocysts are produced following sexual reproduction. Two types of oocysts are produced; thick-walled oocysts, which are the infectious form found in the environment and thin-walled oocysts, which cause autoinfection (Marshall *et al.*, 1997; Monis & Thompson, 2003) (Fig.1.1). Approximately 20% of the zygotes develop into thin-walled oocysts which helps to explain the mechanism of persistent infections (in Acquired Immunodeficiency Syndrome [AIDS] patients) in the absence of successive (thick-walled) oocyst exposures (Current & Garcia, 1991).

Depending on the parasite species, the host and the immunocompetency of the host, the prepatent period, (time between infection and active oocysts shedding), ranges from 1 to 3 weeks, whereas the patent period (duration of oocysts shedding) can range from several days to months or years (O'Donoghue, 1995), demonstrating the potential of this infection to persist (Ramirez *et al.*, 2004).

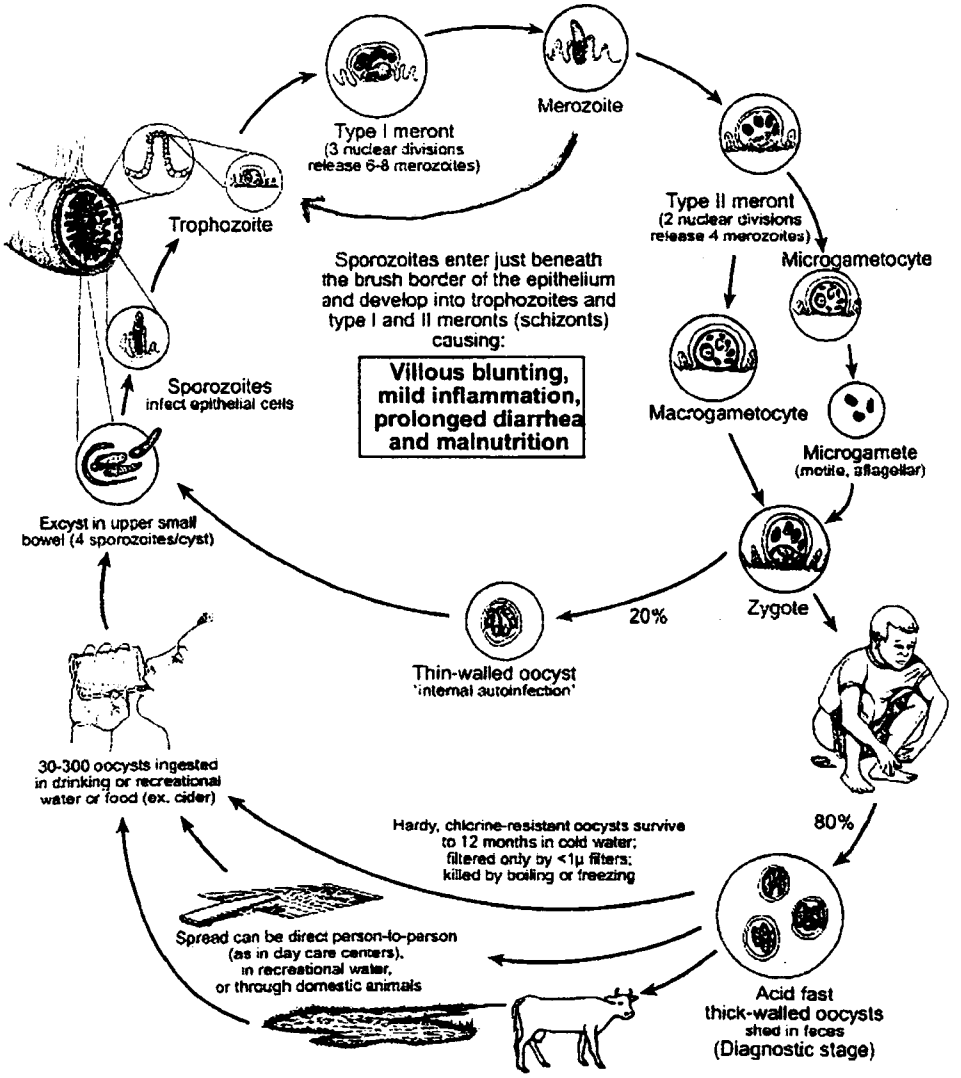


Figure 1.1. Life cycle of *Cryptosporidium hominis / C. parvum.* Taken from Dillingham *et al.* (2000).

1.4. Taxonomy

Cryptosporidium is a genus belonging to Phylum Apicomplexa (which possess an apical complex), Class Sporozosida (which reproduce by asexual and sexual cycles, with oocyst formation), Subclass Coccidiasina (with a life cycle involving merogony, gametogeny and sporogeny), Order Eucoccidiida (in which schizgony occurs), Suborder Eimeriina (in which independent micro- and macrogamy develop), Family Cryptosporiidae (contain four naked sporozoites within oocysts but with no sporocyst) (Levine, 1985; Tzipori & Griffiths, 1998).

Successful *in vitro* cultivation of *Cryptosporidium* spp. (Hijjawi *et al.*, 2002) has shown the non-coccidian, gregarine affinities of the genus which is further supported by recent developmental studies. Similarities between the two groups include a monoxenous life cycle, oocysts with four sporozoites, usually located in the host's gastrointestinal tract and extracellular gamonts or trophozoites (Leander *et al.*, 2003a). This was also supported by phylogenetic studies which confirmed that *Cryptosporidia* were not as closely related to coccidian parasites as originally suspected, but rather were closer to the gregarines (Carreno *et al.*, 1999; Leander *et al.*, 2003b), or to *Plasmodium* spp. rather than *Toxoplasma* and *Eimeria* spp. (Sulaiman *et al.*, 2000).

Cryptosporidium taxonomy has been largely based on a combination of morphology, the site of infection, biological characteristics and the host species range. Initially, species were only recognized if they had clear morphological differences in oocyst size and shape or host specificity. Therefore oocyst structure is usually one of the requirements for establishing a new species. Oocyst morphometrics alone are not entirely adequate for descriptions of new species of *Cryptosporidium,* and other characteristics must be included in the taxonomic description (Xiao *et al.*, 2004). Using morphological criteria and host specificity 19 *Cryptosporidium* species are currently recognized (Table1.1).

Table1.1. **Valid *Cryptosporidium* species based on morphology and host specificity (Fayer *et al.*, 2000; Thompson, 2003; Snelling *et al.*, 2007; Ryan *et al.*, 2008).**

Species name	Type hosts	Oocyst length (µm)	Oocyst width (µm)
C. andersoni	Cattle	6.0-8.1	5.0-6.5
C. baileyi	Chicken	5.6-6.3	4.5-4.8
C. bovis	Ruminants	4.8-5.4	4.2-4.8
C. canis	Dog	3.7-5.9	3.7-5.9
C. fayeri	Red kangaroo	4.5-5.1	3.8-5.0
C. felis	Cat	3.2-5.1	3.0-4.0
C. galli	Birds	8.0-8.5	6.2-6.4
C. hominis	Humans	4.4-5.4	4.4-5.9
C. meleagridis	Turkey	4.5-6.0	4.2-5.3
C. molnari	Marine fish	3.3-5.5	3.0-5.0
C. muris	Mouse	6.6-7.9	5.3-6.5
C. nasorum	Fish	3.5-4.7	2.5-4.0
C. parvum	Mouse	4.5-5.4	4.2-5.0
C. saurophilum	Lizards	4.4-5.6	4.2-5.2
C. scophithalmi	Fish	3.7-5.0	3.0-4.7
C. serpentis	Snakes	5.6-6.6	4.8-5.6
C. suis	Pigs, human	5.1	4.4
C. varanii	Lizards, snake	4.4-5.6	4.2-5.2
C. wrairi	Guinea pig	4.8-5.6	4.0-5.0

Based on the site of infection, *Cryptosporidium* species and genotypes are divided into those that infect the intestine and have small oocysts, e.g. *C. hominis, C. parvum, C. canis, C. felis*; and a smaller group which have larger oocysts and invade gastric or cloacal sites such as *C. serpentis, C. muris* and *C. galli* (Dillingham *et al.*, 2002; Xiao *et al.*, 2004). *C. galli* and *C. varanii*, have been reviewed by Fayer (2004), while *C. galli*

was re-described by Ryan *et al.* (2003). Recently, *C. suis* and *C. bovis* have been described by Ryan *et al.* (2004) and Fayer *et al.* (2005) respectively. Some species are relatively well defined by their morphological and biological characteristics, while others are still enigmatic and appear to represent several cryptic species as revealed by genotyping of different isolates (Morgan *et al.*, 1999b; Xiao *et al.*, 2002a).

At the 6th meeting on Molecular Epidemiology and Evolutionary Genetics in Infectious Disease at the Pasteur Institute in Paris, France, a session was held entitled "The taxonomy of the genus *Cryptosporidium* ". It was suggested that when naming new species of *Cryptosporidium,* four basic requirements should be fulfilled: (i) morphometric studies of oocysts; (ii) genetic characterizations (iii) demonstration of natural and whenever feasible, at least some experimental host specificity, and (iv) compliance with the International Code of Zoological Nomenclature (ICZN) (Xiao *et al.*, 2004).

Various methods have been used to characterize strains and isolates of the genus *Cryptosporidium.* The most commonly used techniques are those based on DNA sequence variations, such as randomly amplified polymorphic DNA (RAPD) PCR, RFLP analysis or sequence comparison of polymorphic genes (e.g. ribosomal DNA or protein coding genes: ß-tubulin, dihydrofolate reductases (dhfr), *Cryptosporidium* Oocyst Wall Protein (COWP), thrombospondin-related adhesion proteins (TRAP-C1 and TRAP-C2), polythreonine (poly(T)), or Heat Shock Protein 70kDa (HSP70) (Morgan *et al.*, 1999b; Morgan *et al.*, 2000b; Xiao *et al.*, 2002a).

The knowledge of the host spectra of different species and genotypes of *Cryptosporidium* is still fragmentary. Reptile and fish isolates of cryptosporidia with low host specificity have been described. However, they do not seem readily to cross the border to warm-blooded hosts, (Fayer *et al.*, 1997; Graczyk *et al.*, 1997). Mammalian species also have varying host specificities: *C. wrairi* seems to be restricted to guinea pigs (Xiao et al., 2002a). In contrast, *C. andersoni* infects cattle (Sreter *et al.*, 2000; Enemark *et al.*, 2002) as well as cervids and rodents (Olson *et al.*, 2004). *Cryptosporidium muris* has been described as infecting mice as well as cattle and humans (Upton & Current, 1985), although early descriptions of *C. muris* might have mistaken this species for *C. andersoni. C. parvum* was originally described as a species with a

broad range of hosts, but genetic analysis has shown that it can be subdivided into several paraphyletic groups (Morgan *et al.*, 1999b). Recently, various separate species have been described, such as *C. felis* which infects cats and cattle (Sargent *et al.*, 1998; Bornay-Llinares *et al.*, 1999), *C. canis* from dogs (Fayer *et al.*, 2001) and *C. hominis* (Morgan-Ryan *et al.*, 2002). Other genotypes with different host preferences are yet to be renamed including, cattle, sheep, goat, pig, marsupial and mouse genotypes of *C. parvum* (Morgan *et al.*, 1999b; Xiao *et al.*, 2002a; Xiao *et al.*, 2004) and thus the taxonomy of the genus *Cryptosporidium* is far from being definitive.

It should be remembered that there is a general problem distinguishing between interspecies differences and intra-specific variability. If all genotypes are given species name the genus will be too complicated and the nomenclature unhelpful.

1.5. Molecular techniques

The introduction of PCR-based assays has had an unprecedented impact on our ability to detect and differentiate *Cryptosporidium* and a number of assays have been developed to identify species, genotypes and subtypes of the parasite, thereby offering an alternative to conventional diagnosis (Caccio, 2003; Caccio *et al.*, 2005). PCR is highly sensitive, rapid and accurate, but false positives can result from laboratory contamination or from the presence of nonviable microorganisms, and false negatives can be caused by PCR inhibitors co-extracted with the nucleic acids (Caccio & Pozio, 2006). The application of PCR in which very small quantities of oocysts and 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* (Balatbat *et al.*, 1996; Webster *et al.*, 1996; Morgan *et al.*, 1998a; Nydam *et al.*, 2002)

1.5.1. Genotypic analysis of *Cryptosporidium* species

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. Five bands were also distinguished by Countour-Clamped Homogeneous Electrophoresis Field (CHEF) in previous studies (Petersen *et al.*, 1992; Steele *et al.*, 1995; Khramtsov *et al.*, 1996). Indeed the *C. parvum* karyotype had remained a rather controversial issue until 1997, when Blunt and others described a new characterization 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% adenine-thymine (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). Furthermore, the low copy and genomic-wide distribution of both 5 S (four to five copies) and the 18S rRNA (five copies) genes is similar to several other apicomplexans, such as *Plasmodium* (Taghi-Kilani *et al.*, 1994; Le Blancq *et al.*, 1997). However, this differs markedly from the usual eukaryotic pattern of hundreds or thousands of tandem copies (Piper *et al.*, 1998). In contrast to other apicomplexan parasites, *Cryptosporidium* species appears to lack plastids (Fayer *et al.*, 1997; Kohler *et al.*, 1997) but the presence of two extra-chromosomal virus-like, double-strand (ds)-RNA structures in the oocysts of *C. hominis* and *C. parvum* were reported by 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 organization of the genome has hindered our understanding 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, the availability of the *Cryptosporidium* genome should provide necessary information for the development of tools for disease prevention and treatment (Liu *et al.*, 1999).

The complete genome sequences of *C. parvum* and *C. hominis* have been obtained and analysed to provide a better understanding of the complicated biology and metabolic activities of these two pathogens (Abrahamsen *et al.*, 2004; Xu *et al.*, 2004). The 9.1 Mbp genome of *C. parvum* is distributed on eight chromosomes and contains an estimated 3807 genes. Structurally, the genome is quite compact relative to the 23-Mb, 14-chromosome genome of the related apicomplexan *Plasmodium falciparum* (Gardner *et al.*, 2002) and in contrast to other apicomplexans including *Plasmodium*, *Toxoplasma* and *Eimeria*. *C. parvum* lacks both apicoplast and mitochondrial genomes and most of nuclear genes that normally control these compartments (Zhu *et al.*, 2000b; Abrahamsen *et al.*, 2004). The compactness of the *C. parvum* genome is shown by its significantly fewer (3807 vs.5268) and shorter (1795 vs.2283 bp, excluding introns) genes than *P. falciparum*. Further in contrast to *P. falciparum*, the *C. parvum* genome is essentially lacking in repetitive DNA (Abrahamsen *et al.*, 2004).

More recently, Xu *et al.* (2004) described the eight-chromosome ~9.2-million-base genome of *C. hominis*. The complement of *C. hominis* protein-coding genes shows a striking concordance with the requirements imposed by the environmental niches the parasite inhabits. Evidence of an apicoplast is absent but genes associated with apical complex organelles are present. The eight chromosomes range from ~0.9 to ~1.4 Mb and exhibit 31.7% GC content compared with 30.3% and 19.4% for *C. parvum* and *P. falciparum* (Gardner *et al.*, 2002) respectively. There are ~3,994 genes in *C. hominis*, in comparison with 3,952 genes in *C. parvum* and 5,268 in *P. falciparum* (Gardner *et al.*, 2002). Comparison of the genomes of *C. hominis* and *C. parvum* show that the two genomes are very similar, exhibiting only 3-5% sequence divergence, with no large insertions, deletions or rearrangements evident. In fact, the gene complements of the two species are essentially identical because the few *C. parvum* genes not found in *C. hominis* are proximal to known sequence gaps. Therefore, the significant phenotypic differences

between these parasites are due to functionally significant polymorphisms in relevant protein-coding genes and to subtle gene regulationary differences as concluded by Xu *et al.* (2004).

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.*, 1997; Morgan *et al.*, 1999a; Xiao *et al.*, 1999b). Ortega and others (1991) were the first to examine genetic polymorphisms and confirmed phenotypic characteristics suggesting that 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-el-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). For example a study analysed 211 faecal specimens using PCR-RFLP analysis of 18S rRNA, COWP and Thrombospondin Related Adhesion Protein-*Cryptosporidium*1 (TRAP-C1) gene fragments. This study found 38% of the samples were *C. hominis* and 62% were *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 were also conducted on *Cryptosporidium* isolates from AIDS patients.

Five patients were infected with *C. hominis* and two with *C. parvum* (Widmer *et al.*, 1998). In each study and at each locus, two electrophoresis profiles were observed and indicated the presence of the two species. Minor differences have been found within *C. hominis* in the 18S rRNA (Xiao *et al.*, 1998), TRAP-C2 (Peng *et al.*, 1997), and poly (T) genes (Widmer, 1998). 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 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 were the 18S rRNA (Xiao *et al.*, 1999b) and the COWP gene (Spano *et al.*, 1997) with the former being the most common target in both prokaryotes and eukaryotes. Other target gene loci also include the Internal Transcribed Spacer1 (ITS1) and the Internal Transcribed Spacer2 (ITS2), (Morgan *et al.*, 1995), the acetyl-CoA synthetase gene, (Black *et al.*, 1996), the dihydrofolate reductase-thymidylate synthase (dhfr) gene, (Gibbons *et al.*, 1998), the 70Kda HSP70 gene, (Sulaiman *et al.*, 2000), the TRAP-C1 and TRAP-C2 genes, (Peng *et al.*, 1997; Spano *et al.*, 1998), poly-T (GP 900) (Spano *et al.*, 1998), RNR, (Widmer *et al.*, 1998), Initiation Translation Factor elF04A, (Spano & Crisanti, 2000) 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.*, 1999b). However, PCR of the COWP, TRAP-C1 and TRAP-C2 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.*, 2001a). While it is evident that some primers will amplify 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 required, 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 characterization of *Cryptosporidium* isolates in clinical and epidemiological studies. However, the limited resolution offered by this method has emphasized 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 couples the advantage 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.

1.5.1.1. The 18S rRNA gene

The small subunit ribosome of *Cryptosporidium* species, like other eukaryotes, has one ribosomal RNA molecule consisting of the 18S rRNA of approximately 2000 nucleotides (Le Blancq *et al.*, 1997). The 18S rRNA gene varies according to the species and genotype of *Cryptosporidium*. The 18S rRNA is approximately 1750 bp in *C. parvum* and the related species *C. hominis*. The longest 18S rRNA gene has been seen in *C. parvum* and *C. hominis* and the shortest gene is in *C. baileyi*. The 18S rRNA is highly polymorphic within the genus and is useful as a target for the identification and differentiation of *Cryptosporidium* species and genotypes. Most of the intra-species differences occur in the first half of the gene (Cai *et al.*, 1992).

1.5.1.2. The GP60 gene

The GP60 gene was known as Cpgp15/45 (Alves *et al.*, 2003), Cpgp 40/15 (Cevallos *et al.*, 2000) or gp60/45/15 (Strong *et al.*, 2000). This gene encodes a 60 kDa glycoprotein that is processed during intracellular parasite development to produce gp15 and gp45 (Strong *et al.*, 2000). The gp15 is present on the entire surface of sporozoites and merozoites and the gp45 is present on the apical complex of the sporozoites and is also localized on the apical complex and entire surface of the merozoites, both of which

are implicated in attachment and invasion of sporozoites to enterocytes (Cevallos *et al.*, 2000; Priest *et al.*, 2000).

The molecule is encoded by a single copy gene which does not have introns. Also, no similarity was seen in the deduced amino acids sequences between the gp45 and gp15 fragments (O'Connor *et al.*, 2002). An important feature of the GP60 gene is its high degree of sequence polymorphism, particularly among *C. hominis* isolates. Sequence analysis of this gene divides both *C. hominis* and *C. parvum* into at least eight allele families each with several sub-genotypes. Members of different allele families differ from each other extensively in the primary sequence but within each allele family, sub-genotypes differ from each other mostly in the number of trinucleotide (TCA) repeats (Strong *et al.*, 2000; Alves *et al.*, 2003; Xiao, 2003). There are nearly 100 GP60 sub-genotypes of *C. hominis* and *C. parvum*, indicating a very high resolution for this technique. Unfortunately this tool does not clearly divide *C. hominis* and *C. parvum* into two separate groups. Therefore more sub-genotyping tools are necessary to increase the resolution of current sub-genotyping technique as suggested by Xiao *et al.* (2003).

1.5.1.3. The HSP70 gene

The heat shock protein (HSP) gene belongs to a multi-gene family that is highly conserved across the prokaryotes and eukaryotes. Under normal conditions these proteins function as molecular chaperons for facilitating the folding of proteins in secretion and transport, while under environmental stress their expression is up-regulated and is involved in the protection of the cells, (Ellis & Van der vies, 1991; Gething & Sambrook, 1992; Gupta & Golding, 1993; Gupta *et al.*, 1997). The polymorphic nature of the HSP70 gene sequences was realized by Sulaiman *et al.* (2000) who found that the HSP70 gene was a good target for genotyping based on its high level of heterogeneity spread over the entire sequence of various *Cryptosporidium* isolates from human and animal hosts

1.5.2. Phylogeny of *Cryptosporidium*

Molecular data based on DNA has become an important tool in evolutionary studies. Evolutionary changes in morphological and physiological characteristics are so complex and do not produce a clear-cut picture of evolutionary history and are largely impeded 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 (Li, 1997).

Molecular phylogenetic analyses of the Apicomplexa that 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 other major apicomplexan clades containing the coccidians, adeleids, piroplasms and haemosporinids, indicating that *Cryptosporidium* has a closer phylogenetic association with the gregarines than the coccidians. Perhaps the most interesting aspect of a link between *Cryptosporidium* and the gregarines is suggested by phylogenies derived from small subunit ribosomal DNA sequences indicating that *Cryptosporidium* parasites might be most closely related to some of the earliest diverging apicomplexan parasites, the archigregarines (Leander & Keeling, 2004). The understanding of a shared ancestry for *Cryptosporidium* parasites with gregarines will have a significant impact on how we understand and deal with the basic biology of *Cryptosporidium* parasites (Leander *et al.*, 2006) and on how we view the 'primitive' gregarines found in many invertebrates. It might be possible to exploit this relationship at a practical level in the future, by using gregarine parasites as more accessible laboratory

models for cryptosporidiosis, particularly for drug discovery. Are there *Cryptosporidium* species waiting to be discovered (or biologically similar apicomplexan parasites, perhaps an archigregarine) that infect the alimentary tract of invertebrates? (Barta & Thompson, 2006).

Zhu *et al*, (2000) 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.*, 2000a) (Figure 1.2). It appears that there may be genuine differences in the genome and the biology of the genus that set it apart from its closest relatives (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.*, 2000a). 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.*, 2000a).

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 eucoccidia 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). The non-coccidian, gregarine affinities of *Cryptosporidium* are further supported by recent developmental studies. The similarities between the method of feeding in cryptosporidians and that seen in many gregarines are striking (Thompson *et al.*, 2005). Also, stages of *Cryptosporidium* observed in cell-free culture show remarkable similarities to those seen in the life cycles of some gregarines (Hijjawi *et al.*, 2002; Hijjawi *et al.*, 2004). The occurrence of predominantly extracellular

stages in the life cycle that can be completed in cell-free culture has demonstrated that *Cryptosporidium* is not as obligatory an intracellular organism as previously thought. Perhaps predictably, the greatly reduced biochemical repertoire retained by *Cryptosporidium* species after loss of both a functional mitochondrion and an apicoplast requires a nutrient-rich environment from which to salvage its metabolic needs.

Results of molecular and phylogenetic 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 (Morgan *et al.*, 1999a; Xiao *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,* and *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 analysis of two single-copy genes; the HSP70 and the COWP genes (Xiao *et al.*, 2000b).

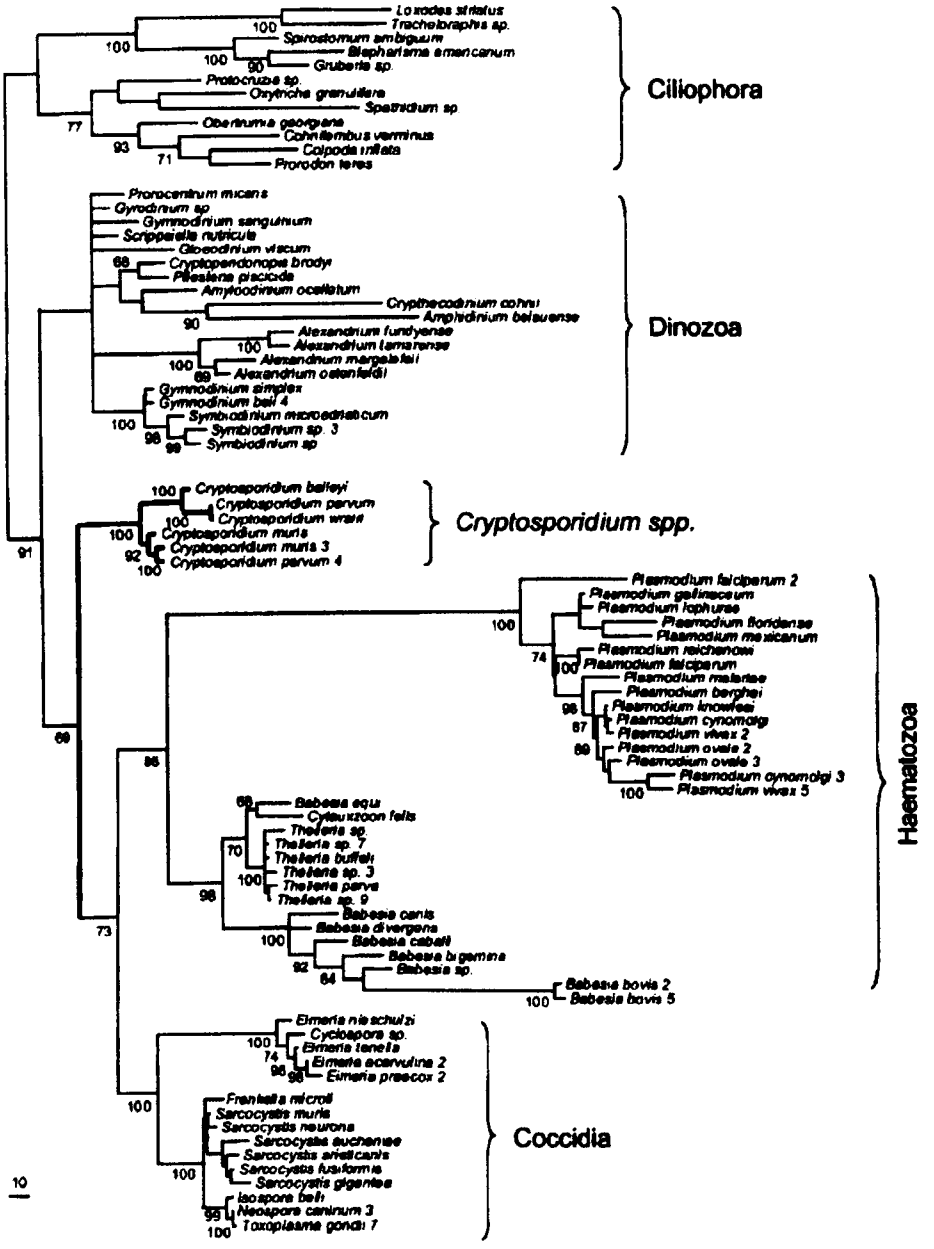


Figure 1.2. Maximum Parsimony tree from SSU rRNA dataset containing 52 complete sequences within the phylum Apicomplexa using Dinozoa and Ciliophora as out-groups to root the tree. Note the *Cryptosporidium* species emergence as a sister group of the Haematozoa rather than a number of the coccidea. From Zhu *et al.* (2000b).

1.6 *Cryptosporidium* species

Cryptosporidium species infect a wide range of vertebrates, including mammals, birds, reptiles, fish and amphibians, (Monis & Thompson, 2003; Fayer, 2004). The following is a review of the species infecting these hosts.

1.6.1. *Cryptosporidium* species of mammals

Mammals represent the largest group of animas known to be infected with *Cryptosporidium* spp., probably due to the greater number of studies as a result of the perceived importance of such animals. Most infected animals are found in the orders Artiodactyla, Primates and Rodentia. Since the *Cryptosporidium* spp. from the majority of these animals have been identified by oocyst morphology alone, with little or no host specificity and/or molecular data to support identification, it is not clear how many of the reported isolates are actually *C. parvum* or other species, (Fayer, 2004; Joachim, 2004).

1.6.1.1. *Cryptosporidium muris*

In 1907 Ernest Edward Tyzzer described a protozoan parasite that he frequently observed in the gastric glands of laboratory mice but not wild mice (Tyzzer, 1907). The asexual meront stages contained six merozoites each with a distinct nucleus. Sexual stages were observed and measured. Spore (oocyst) formation was described with large oocyst measuring about 7 by 5 μm. Subsequently nearly all 'large' oocysts of *Cryptosporidium* from mammals or those found in the stomach were called *C. muris* or *C. muris*-like (Fayer, 2003). This species can infect mice, guinea pigs, rabbits, dogs, and cats.

Recent studies have shown *C. muris* to be capable of infecting a wide range of additional hosts including hamsters, squirrels, Siberian chipmunks, wood mice, bank voles, Bactrian camels, mountain goats, humans and cynomolgus monkeys (Anderson, 1991a; Xiao *et al.*, 2004).

1.6.1.2. *Cryptosporidium andersoni*

C. andersoni infects the abomasums of cattle and produces oocysts morphologically similar to but slightly smaller than those of *C. muris* (Lindsay *et al.*, 2000), and named after Bruce Anderson, University of Idaho, the original finder of the parasite. Oocysts are passed fully sporulated, lack sporocysts, are ellipsoid measuring 7.4 x 5.5μm (6.0 to 8.1 x 5.0 to 6.5) with a length/width ratio of 1.35. Unlike those of *C. muris*, oocysts of *C. andersoni* are not infectious for out-bred, inbred immunocompetent or immunodeficient mice, nor are they infectious for chickens or goats. *C. andersoni* was recognized early on to be poorly infective not only to non-bovine hosts but also to cattle. Thus oocysts derived from cattle previously identified as *C. muris*-like, were not infectious for mice or even other cattle (Anderson, 1991a).

1.6.1.3. *Cryptosporidium parvum*

The most frequently reported species in mammals *C. parvum* was first found in mice, (Tyzzer, 1912). It was differentiated from *C. muris* based on its smaller oocyst size and its location only in the villi of the small intestine, most frequently near the tips. Oocysts were ovoidal or spherical and did not exceed 4.5 μm in diameter.
Over 150 species of mammals have been identified as hosts of *C. parvum* or *C. parvum*-like parasites. However, most descriptions have been based solely on microscopy, with no careful morphometric measurements or genetic or other biological data. Recent molecular characterizations have shown that there is extensive host adaptation in *Cryptosporidium* evolution. *C. parvum* is known to infect mainly ruminants (cattle, sheep, goats, and deer) and humans (Xiao *et al.*, 2004).

1.6.1.4. *Cryptosporidium canis*

Cryptosporidium oocysts have been observed in the faeces of dogs worldwide. Based on its ability to infect humans and bovines but its inability to infect mice as well as significant genetic differences from other *Cryptosporidium* spp., the parasite was named *C. canis*. Confirmed *C. canis* infections have been found in dogs, coyotes, foxes and humans (Fayer *et al.*, 2001).

1.6.1.5. *Cryptosporidium felis*

C. felis was initially reported with a description of the oocyst from the faeces, basic observation of endogenous development and some work on host specificity and pathogenicity (Iseki, 1979; Fayer *et al.*, 1997). Even though the validity of *C. felis* was in doubt for some years, recent molecular characterizations at the SSU rRNA, ITS-1, HSP70, COWP, and actin loci support the concept of *C. felis* as a valid species. Confirmed *C. felis* infections have been found in cats, humans, and cattle (Xiao *et al.*, 2004).

1.6.1.6 *Cryptosporidium wrairi*

Cryptosporidium wrairi from the guinea pig (*Cavia porcellus*) was named as an acronym for the Walter Reed Army Institute of Research (Vetterling *et al.*, 1971). Usually small guinea pigs (weighing 200 to 300 g) were found to be infected. Cross-transmission studies suggested that *C. parvum* and *C. wrairi* might actually be the same species. More recently molecular characterizations have identified significant differences between *C. parvum* and *C. wrairi* at multiple genetic loci. These combined data along with the fact that naturally occurring *C. wrairi* infections have been found only in guinea pigs strongly suggest that this organism is a different species from *C. parvum* (Xiao *et al.*, 2004).

1.6.1.7. *Cryptosporidium hominis*

Cryptosporidium parasites infecting humans, previously designated *C. parvum* human genotype, genotype 1, or genotype H, have been delineated as a separate species, *C. hominis,* based on molecular and biological differences (Morgan-Ryan *et al.*, 2002). *C. hominis* is morphologically identical to *C. parvum*. Unlike *C. parvum*, *C. hominis* is traditionally considered non infective for mice, rats, cats, dogs, and cattle. Pathogenicity studies with gnotobiotic pigs have shown the prepatent period to be longer than for *C. parvum* 8.8 and 5.4 days, respectively. There appear to be distinct differences in oocyst shedding patterns between *C. hominis* and *C. parvum* in humans. A study in the United

Kingdom revealed that *C. hominis* was detected in a significantly greater proportion of samples with larger numbers of oocyst whereas *C. parvum* was detected in a significantly greater proportion of the samples with small numbers of oocysts (McLauchlin *et al.*, 1999).

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, opossums (two genotypes), ferrets, foxes, deer (two genotypes), muskrats (two genotypes), squirrels, bear and deer mice (Xiao *et al.*, 2002b). The genetic distances between these *Cryptosporidium* parasites are greater than or comparable to those between established intestinal *Cryptosporidium* species. Limited cross-transmission studies have shown biological differences among some of the genotypes (Enemark *et al.*, 2003).

1.6.1.8. *Cryptosporidium bovis*

Oocysts of *C. bovis*, previously identified as *Cryptosporidium* genotype Bovine B, are morphologically indistinguishable from those of *C. parvum*. They are excreted fully sporulated and contain 4 sporozoites, but lack sporocysts. Oocysts measure 4.76-5.35 μm x 4.17-4.76 μm, with a length-to-width ratio of 1.06. This new species, which mainly infects bovine intestine, has been found in 10 of 162 calves aged 9 to 11 months on a beef farm in Maryland (Fayer *et al.*, 2005). *C. bovis* can be detected from three weeks onwards and it has never been detected in humans (Slapeta, 2006). It was found to be the dominant species in 1-2-year-old cattle (Fayer *et al.*, 2006).

1.6.1.9. *Cryptosporidium fayeri*

More recently, oocysts of a new species of *Cryptosporidium* have been found in the faeces of the red kangaroo (*Macropus rufus*) by Ryan and others (2008). Oocysts of the new species, named *C. fayeri*, are passed fully sporulated, lack sporocysts, and

measure 4.5-5.1 μm x 3.8-5.0 μm. Multi-locus analysis of numerous unlinked loci demonstrated this species to be distinct from *C. parvum* (Ryan *et al.*, 2008).

1.6.2. *Cryptosporidium* species of birds

Cryptosporidium infections have been found in over 30 species of birds including domesticated chickens, turkeys, ducks, geese, quails, pheasants, peacocks and a wide variety of wild and captive birds (de Graaf *et al.*, 1999; Morgan *et al.*, 1999b; Sreter *et al.*, 2000; Sreter & Varga, 2000; Joachim, 2004; Xiao *et al.*, 2004). Significantly, only three avian *Cryptosporidium* spp have been named. *C. meleagridis* infects the small and large intestine with an oocyst measurement of 4.5 x 4 μm (Salvin, 1955). *C. baileyi* is also found in the small and large intestine but can infect the respiratory tract. The oocysts from this species are significantly bigger than those from *C.meleagridis* with measurements of 6.2 x 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 x 6.3 μm (Xiao *et al.*, 2004). However, recent molecular characterization of isolates of *Cryptosporidium* from various species of birds has identified two new genotypes/species of *Cryptosporidium* from finches and a black duck (Morgan *et al.*, 2001).

1.6.3. *Cryptosporidium species of reptiles*

Cryptosporidium spp. have been reported in over 57 different reptilian species, including 40 species of snakes, 15 species of lizards and 2 species of tortoises (O'Donoghue, 1995). Among reptiles, snakes are affected most severely by cryptosporidiosis due to the chronic and detrimental nature of the infection. *C. serpentis* was isolated and named by Levine in 1980 (Levine, 1980). It has distinguishable oocysts (6.2 x 5.3 μm), which differentiate it from the other species infecting reptiles. This species was for quite some time the only species identified in reptiles. Recently *C. saurophilum* described and named following an extensive study of the faeces from 220 wild and captive lizards of 67 different species (Koudela, 1998). More recently this

parasite was found among 9 of 24 *Cryptosporidium* isolates from monitors, iguanas and geckos (Xiao *et al.*, 2004).

1.6.4. *Cryptosporidium* species of fish

Two named species of *Cryptosporidium* have been found in fish: *C. nasorum* (Hoover, 1981) and *C. molnari* (Alvarez-Pellitero & Sitja-Bobadilla, 2002). Reports of infection with *C. nasorum* have been described in captive or ornamental fish (Landsberg, 1986). Parasites have been detected in the stomach, intestinal tract and faeces of the fish. No measurements of viable oocysts were available (Xiao *et al.*, 2004). The second species is *C. molnari* in which most of its stages were located at the surface of epithelial cells in the stomach with oocysts measuring 4.7 x 4.4 μm (Alvarez-Pellitero & Sitja-Bobadilla, 2002). Recently, *Cryptosporidium scophthalmi* has been described from the turbot *Scophthalmus maximus* L., sampled from different farms in Spain. This parasite was found mainly in the intestinal epithelium and very seldom in the stomach. Oocysts were spherical, with 4 naked sporozoites and a resedium, and measured 3.7-5.03 x 3.03-4.69 μm (Alvarez-Pellitero *et al.*, 2004).

1.7. 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 variation in the phenotypic expression of the different isolates. These differences have been recognized based on infectivity for other animals (O'Donoghue, 1995), pathogenicity (Fayer & Ungar, 1986) and antigenicity (McDonald *et al.*, 1991). A study showed differences in the mean oocyst infective doses for humans between three different *Cryptosporidium* isolates (DuPont *et al.*, 1995). In a further 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. Recently, genetic characterization using specific and reliable techniques such as PCR-RFLP and sequence analyses have been employed to differentiate between *Cryptosporidium* isolates and to confirm the validity of the species within this genus.

Analysis of the 18S rRNA gene has revealed the existence of different genotypes of *C. parvum* arbitrary assigned 'human', 'bovine', 'cattle', 'pig', 'cat', 'mouse', 'dog', 'monkey', 'ferret' and 'masurpial' types (Xiao *et al.*, 1999b). Sequence analysis of additional genes such as ribosomal internal transcribed spacer (ITS rDNA) regions, COWP gene, dihydrofolate reductase-thymidylate synthase (dhfr-ts), TRAP-C1 and C2, and HSP70 locus have differentiated between *Cryptosporidium* species (*C. parvum, C. hominis, C. meleagridis, C. baileyi, C. muris, C. felis, C. serpentis, C. wrairi*) and *C. parvum* genotypes (pig, ferret, mouse and monkey). For instance, phylogenetic analysis based on the 18S rRNA and HSP70 genes indicate that *Cryptosporidium* forms two major groups. *C. muris* and *C. serpentis* constitute one group and *C. hominis*, *C. parvum*, *C. baileyi*, *C. felis*, *C. canis* and *C. meleagridis* form the other (Xiao *et al.*, 1999a; Sulaiman *et al.*, 2000). *C. felis*, *C. canis* and *C. baileyi* are variants from the main clades of *C. parvum* and thus have been classified as different species. Whilst *C. meleagridis* and *C. wrairi* cluster with *C. parvum* they are still considered distinct species based on biological or phenotypic characteristics.

1.8. *Cryptosporidium* species and genotypes which infect humans

1.8.1. *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, (Fayer *et al.*, 2000; McLauchlin *et al.*, 2000).

The two leading causes of human cryptosporidiosis are *C. hominis* and *C. parvum*, previously referred to as *C. parvum* genotypes 1 and 2 but now recognized as two distinct species. Morphologically, *C. hominis* and *C. parvum* oocyst isolates are indistinguishable from each other both measuring 4.4-5.4 μm. Other than humans, *C. hominis* can infect neonatal pigs, dugongs and lambs (Morgan-Ryan *et al.*, 2002), whereas *C. parvum* oocysts are able to infect mice, rats, dogs, cats or calves.

C. hominis is usually the predominant species in humans globally with the exception of European countries (Xiao & Ryan, 2004). Studies in the United States,

Australia, Kenya, Thailand, South Africa and Malawi have indicated that *C. hominis* is responsible for the majority of cases of human cryptosporidiosis (Gatei *et al.*, 2003; Peng *et al.*, 2003; Xiao, 2003).

A difference was observed between the *Cryptosporidium* species from rural and urban isolates, with *C. hominis* dominant in the urban region, whereas *C. parvum* was prevalent in rural New Zealand (Learmonth *et al.*, 2004). There are also distinct geographical and temporal variations in the distribution of *C. parvum* and *C. hominis* infections in humans. In patients in the United Kingdom *C. parvum* was more common during spring whereas *C. hominis* was more common in late summer and autumn in those with a history of foreign travel, (McLauchlin *et al.*, 2000). A shift in human infection from predominantly *C. parvum* in the spring to *C. hominis* in the autumn has also been reported in New Zealand (Learmonth *et al.*, 2003).

1.8.2. *Cryptosporidium parvum*

C. parvum, which was previously known as the bovine genotype, is the most frequently reported species in mammals. 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 characterization, 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 sequence 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, marsupials, monkey, muskrat, skunk, cattle and ferret (Xiao *et al.*, 2002b). *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).

Most European studies have reported that *C. parvum* is more commonly detected in patients with cryptosporidiosis than *C. hominis* (McLauchlin *et al.*, 1999; Alves *et al.*, 2001; Guyot *et al.*, 2001; Pedraza-Diaz *et al.*, 2001b).

Outbreaks caused by *C. parvum* have all been epidemiologically linked to contamination from, or direct contact with animals, such as the Maine fresh pressed apple cider (Millard *et al.*, 1994), the Pennsylvania rural family and the Minnesota zoo outbreaks between 1995 and 1997 (Sulaiman *et al.*, 1998).

The ubiquitous geographic distribution and wide host range of *C. parvum* and its ability to cause infection at relatively low doses enhances the potential for waterborne transmission. As infected livestock have considerable potential for contaminating aquatic environments, agricultural practices are an important source of oocyst contamination (Carey *et al.*, 2004).

1.8.3. *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 & Varga, 2000). It was first described in 1955 in association with illness and death in turkeys (Salvin, 1955). Oocysts are morphologically similar to *C. parvum*, *C. felis*, and *C. wrairi* (Fayer *et al.*, 2000).

C. meleagridis in humans has been associated with gastrointestinal symptoms, and whether simultaneous infections of the respiratory tract occur as well, is not clear (Akiyoshi *et al.*, 2003). Recent studies have shown *C. meleagridis* to be the third most common *Cryptosporidium* parasite in humans in both immunocompetent and immunocompromised individuals (McLauchlin *et al.*, 2000; Morgan *et al.*, 2000a; Xiao *et al.*, 2001). 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 prevalences as high as the bovine genotype of *C. parvum* (Xiao *et al.*, 2001), again it was reported from HIV-infected individuals from Thailand (Gatei *et al.*, 2002b), Portugal (Matos *et al.*, 2004) and immunocompromised patients from France (Guyot *et al.*, 2001).

Human infection with *C. baileyi,* the second avian species, seems to be rare. In one report from the Czech Republic, oocysts of *Cryptosporidium* spp. whose morphology and measurements corresponded with those of the species *C. baileyi* were found in the

stool of an immunodeficient patient (Ditrich *et al.*, 1991). However this infection may have occurred due to disruption of the immune system by HIV and to immunosuppressive therapy undertaken after allogeneic kidney transplantation as suggested by Ditrich *et al.*, (1991).

1.8.4. *Cryptosporidium felis* and *Cryptosporidium canis*

Cryptosporidium felis was named for its host in 1979, based on studies which indicated *Cryptosporidium* oocysts from cats could not be transmitted to rodents (Iseki, 1979). In 1998 both morphological and molecular evidence was obtained that supported *C. felis* as a distinct species (Sargent *et al.*, 1998). In 1999, molecular evidence suggested that *C. felis* was not only infectious for cats but also for cows (Bornay-Llinares *et al.*, 1999) and also for immunosurpressed humans (Pieniazek *et al.*, 1999). Subsequently, the infection rates of *C. felis* in different areas were reported: 22.7% of *Cryptosporidium* infections identified in HIV positive persons from Switzerland and USA (Morgan *et al.*, 2000a), 1.2% of infections in children from Peru (Xiao *et al.*, 2001), in an HIV positive patient from Italy (Caccio *et al.*, 2002), 0.24% of patients from UK of which 0.12% were immunocompetent patients (Pedraza-Diaz *et al.*, 2001a) and 10% of patients with HIV from Portugal (Matos *et al.*, 2004).

The first isolation of *C. canis* from an HIV positive patient was reported by Pieniazek *et al.*, (1999). In 2001, molecular evidence combined with cross transmission studies demonstrated that the newly named species *C. canis* from dogs was also infectious for humans and cattle (Fayer *et al.*, 2001). In a study on *Cryptosporidium* parasites from patients in Peru, analysis of 951 *Cryptosporidium*-positive specimens from 300 HIV positive patients using an 18S rRNA-based PCR-RFLP tool identified 6 genotypes of *Cryptosporidium* including *C. canis* (4.0%) (Cama *et al.*, 2003). The first report of *C. canis* from the faeces of Thai patients (HIV-infected) was reported by Gatei *et al.*, (2002b) who showed two cases in 36 samples.

1.8.5. *Cryptosporidium muris*

Tyzzer described and named the original *Cryptosporidium* species, *C. muris*, which he observed in the stomachs of the common mice in 1907 (Tyzzer, 1907). Subsequently nearly all large oocysts of *Cryptosporidium* from mammals or those found in the stomach were called *C. muris* or *C. muris*-like. In 2000 on the basis of molecular techniques two distinct genotypes of this parasite were identified, one in cattle and camels and another in mice, hamsters, rock hyrax and camels (Morgan *et al.*, 2000c). In humans, six different cases of infection with this species have been identified since its identification. 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 others were in HIV-infected adults in Kenya and Peru (Gatei *et al.*, 2002a; Tiangtip & Jongwutiwes, 2002; 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). One new genotype of *C. muris* infection was reported in an immunocompromised patient in France based on sequence analysis of 18S rRNA gene (Guyot *et al.*, 2001).

1.9. *Cryptosporidium* species and genotypes which infect ruminants

Ruminants are mostly infected with two species of *Cryptosporidium*: *C. parvum* which colonizes the small intestine and *C. andersoni* present in the abomasums of these animals (Olson, 2003).

1.9.1. *C. parvum*

Infection with *C. parvum* in ruminant species is typically symptomatic in the young population. Among cattle, calves are susceptible to infection shortly after birth and remain so for several months (Xiao & Herd, 1994). *C. parvum* mostly infects the intestine of neonatal calves, has high infection rates and intensities and has an oocyst shedding duration of only 1-2 weeks. (Anderson, 1991a; Anderson, 1991b). Studies have shown a wide range of oocyst shedding dynamics depending on the age, clinical situation, and breeding system of the animals (Maldonado-Camargo *et al.*, 1998). Studies on cryptosporidiosis in cattle and goats in Sri Lanka (Noordeen *et al.*, 2000) and Poland

(Majewska *et al.*, 2000) showed that oocyst shedding was significantly higher in neonatal and young animals compared with adult individuals. Infection can be spread animal-to-animal by the faecal-oral route, usually when animals are housed together in an overcrowded environment but contamination of udders and water supplies by faeces is another common source of transmission in livestock (Ramirez *et al.*, 2004). Transmission of *C. parvum* from calves to humans is also established (Miron *et al.*, 1991; Lengerich *et al.*, 1993). It is well known that most data on the prevalence of *Cryptosporidium* infection in farm animals concern cattle. In comparison there is less information on the occurrence of cryptosporidiosis in sheep and goats (Casemore *et al.*, 1997). This is surprising since the infection in these animals is common and may be severe, often causing death of diarrhoeic lambs and kids (Tzipori *et al.*, 1981; Kaminjolo *et al.*, 1993; Xiao *et al.*, 1993; Olson *et al.*, 1997; Vieira *et al.*, 1997). For example, an atypical outbreak of caprine cryptosporidiosis occurred in the Sultanate of Oman. It occurred in goats ranging in age from two days to adulthood, on a well-managed closed farm. None of the other animals on the farm, including sheep, cows and buffalo were affected. Morbidity approached 100 per cent in goats less than six months of age. Despite intensive supportive care, 238 goats died, ranging in age from two days to over one year. Cryptosporidia were detected in large numbers in the intestinal contents of dead animals and in faecal smears of animals with diarrhoea (Johnson *et al.*, 1999). A study in Poland by Majewska *et al.*, (2000) to evaluate the prevalence of *Cryptosporidium* in sheep and goats, concluded that *C. parvum* infection was identified in 16 of 159 sheep (10.1%) and that the intensity of infection was higher in lambs than in sheep. No *Cryptosporidium* oocysts and/or coproantigen were detected in faecal specimens taken from goats. *Cryptosporidium* isolates from different hosts demonstrated that *C. parvum* isolated from sheep displayed the calf genotype which is common to cattle, goats and humans and it is geographically widespread (Morgan *et al.*, 1998b). Thus, sheep infected with *C. parvum* may be a source of infection both for other farm animals and for humans.

1.9.2. *Cryptosporidium andersoni* and other species

C. andersoni was reported in poorly growing Idaho cattle and has now been identified on most continents. This species infects the abomasums of juvenile and adult cattle, has low infection rates and intensities, and has a long oocyst shedding duration of months to years (Lindsay *et al.*, 2000). The oocysts of *C. andersoni* are morphologically similar to but slightly smaller than those of *C. muris* (Lindsay *et al.*, 2000). Unlike those of *C. muris*, oocysts of *C. andersoni* were not infectious for outbred, inbred immunocompetent, or immunodeficient mice, nor were they infectious for chickens or goats. *C. andersoni* was recognized early on to be poorly infective not only to nonbovine hosts but also to cattle (Xiao *et al.*, 2004).

Some other species were recently reported in calves. For the first time the natural infection and completion of the life cycle of *C. hominis* in two cases of cattle from Scotland, UK has been reported (Smith *et al.*, 2005).

More recently a new species of *Cryptosporidium* in calves, *C. bovis*, has been described (Fayer *et al.*, 2005). Oocysts of *C. bovis*, previously identified as *Cryptosporidium* genotype Bovine B, are morphologically indistinguishable from those of *C. parvum*. They are excreted fully sporulated and contain four sporozoites, but lack sporocysts. Oocysts measure 4.76-5.35 μm (mean = 4.89 μm) x 4.17-4.76 μm (mean = 4.63 μm) with a length-to-width ratio of 1.06 (n = 50). Oocysts were not infectious for neonatal BALB/ c mice but were infectious for two calves that were previously infected with *C. parvum*. Oocysts were not infectious for two experimentally exposed lambs less than one week of age and were not detected in 42 lambs 2-3 months of age but were detected in a two week-old lamb (Fayer *et al.*, 2005).

Slapeta (2006), proposed the name *Cryptosporidium pestis* n. sp. for the species formerly recognized as the 'bovine genotype' of *C. parvum*. He concluded that the name *C. parvum* should be used in the context for which it was originally described: for the mouse species that does not infect humans or domestic cattle (Slapeta, 2006).

In response to the proposal of Slapeta (2006), Xiao *et al* (2007) concluded that the description of *Cryptosporidium pestis* did not follow most of the recommended criteria

for naming *Cryptosporidium* species, thus the new species name should be considered invalid.

1.10. Epidemiology

The identification of *Cryptosporidium* spp. in approximately 3800 stool samples collected worldwide has shown that *C. hominis* and *C. parvum* are the major causes of human cryptosporidiosis, accounting for more than 90% of cases (Caccio & Pozio, 2006). Several factors facilitate the transmission of *Cryptosporidium* and explain its propensity to cause large-scale outbreaks of diarrhoea. Firstly, *Cryptosporidium* can infect many mammalian species. It is identified frequently in farm animals, particularly calves, as well as in domesticated animals. Secondly, the organism is very hardy and can survive in moist environments for prolonged periods of time which may reach 6 months, after that infectivity rapidly decreases (Fayer *et al.*, 1998). Studies have shown that oocysts can survive for months in cold water and that chlorination methods used in water treatment plants are insufficient to eliminate the parasite from drinking water in a reliable and effective manner. Thirdly, numerous species can infect humans. In recent years, population genetic and epidemiologic studies have demonstrated that the *Cryptosporidium* genus is composed of a large number of species that were not previously thought to infect humans. Fourthly, the infectious dose is very small and infected individuals excrete large numbers of oocysts (Huang *et al.*, 2004).

Globally, *Cryptosporidium* has now been recognized 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).

Contaminated drinking water frequently constitutes a source of infection for humans and animals and can lead to endemic infections of spectacular dimensions. Other sources for infection are public swimming pools (Puech *et al.*, 2001) and foodstuff (Laberge & Griffiths, 1996; Fricker & Crabb, 1998; Fretz *et al.*, 2003). Close contact between pets and their owners is probably associated with an increased risk of infection, at least for immunocompromised patients (Glaser *et al.*, 1998; Pieniazek *et al.*, 1999;

Morgan *et al.*, 2000a). Insects such as *Musca domestica* can act as mechanical vectors (Clavel *et al.*, 2002). Domestic and wild animals can contribute to the contamination of surface waters with oocysts (Graczyk *et al.*, 1997), especially when a large number of susceptible hosts e.g. neonates which excrete oocysts are present (Bodley-Tickell *et al.*, 2002). Infectivity studies in animals and humans have shown that as few as one to 10 oocysts can cause infection and that humans can excrete as many as 10^8 oocysts in a single day (Chappell & Okhuysen, 2002). Furthermore, unlike other mammals, humans are susceptible to repeated infections. When shed, *Cryptosporidium* oocysts are infectious. Unlike related coccidian parasites *Cryptosporidium* oocysts do not require a sporulation phase outside the host before becoming infectious (Huang *et al.*, 2004).

There has been a steady accumulation of epidemiological data during recent years on *Cryptosporidium* isolates around the world. Although it is difficult to compare the prevalence and incidence of human cryptosporidiosis from the available data because the examined populations as well as the techniques used to detect infection vary between studies and comparative data are not readily available (Griffiths, 1998). Nevertheless, differences in epidemiology, infectivity and symptom severity are now being shown between different species in the same geographical zone (Hunter *et al.*, 2004).

There are discrepancies in the prevalence between different surveys which were done on human cryptosporidiosis. In the Republic of Korea, the prevalence of cryptosporidiosis varied according to the localities. For example, Lee *et al.*, (2005) reported a prevalence of 1% (among non-HIV patients), whereas Yu *et al.*, (2004) reported a prevalence of 3.3% among the villagers in several rural areas. Also Park *et al.*, (2006), Seo *et al.*, (2001), and Chai *et al.*, (1996) reported 1.5%, 1.9% and 7.9% positivity rates for human cryptosporidiosis, respectively. In Tanzania, Houpt *et al.*, (2005) described a prevalence of 17.3% amongst HIV patients. In Guinea Bissau, *Cryptosporidium* sp. had a prevalence of 7.7% and was the second most common parasite with a marked seasonal variation, with peak prevalence found consistently at the beginning of or just before the rainy seasons, i.e., from May to July (Perch *et al.*, 2001). Recently a study from Iran by Mirzaei, (2007) to evaluate the prevalence of *Cryptosporidium* spp. infection in diarrhoeic and non-diarrhoeic humans showed that the

overall prevalence of *Cryptosporidium* infection was 10.8%, but the prevalence (25.6%) in diarrhoeic humans was higher than that (3.7%) in non-diarrhoeic humans and the association between *Cryptosporidium* spp. infection and occurrence of diarrhoea was significant ($P<0.05$) (Mirzaei, 2007).

Though humans seem to be susceptible at any time in their lives (Fayer & Ungar, 1986), previous studies have shown the highest incidence of cryptosporidiosis is in children younger than 2 years (Tumwine *et al.*, 2003; Steinberg *et al.*, 2004). However in a review on *Cryptosporidium* infections in Saudi Arabia and neighbouring countries by Areeshi *et al* (2007), the incidence of cryptosporidiosis was higher in children under 7 years old, and in Iran, the incidence of the infection was higher in children under 15 years old (Mirzaei, 2007). However, more studies need to be done in order to confirm this hypothesis.

It has been concluded that the prevalence of *Cryptosporidium* infection differs from region to region and also from report to report from the same area. These differences have been attributed to variations in the type of patients studied, whether they are hospitalized 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 (Tzipori, 1988), geographic variation and socio-economic factors (Casemore *et al.*, 1997).

1.11. Risk Factors

Travellers diarrhoea is common in visitors from developed countries to less affluent nations of the world. The rates vary according to the region of the world visited and are the highest in visitors to tropical and semitropical nations during warm rainy months (Steffen *et al.*, 2004; Al-Abri *et al.*, 2005). Very mild infections may be under-diagnosed and may cause typical traveller's diarrhoea (Goodgame, 2003). The most common intestinal protozoa infecting travellers are *Giardia lamblia*, *Cryptosporidium* spp, and *Entamoeba histolytica/dispar*, although a smaller proportion of infections are

due to *Microsporidia* and to *Isospora*. There are some studies of cryptosporidiosis in the UK and Switzerland of those with a history of overseas visits (McLauchlin *et al.*, 2000; Pedraza-Diaz *et al.*, 2001b; Glaeser *et al.*, 2004).

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). Gendrel *et al.*, (2003) concluded that diarrhoea caused by *Cryptosporidium* spp. may be severe in malnourished or immunodeficient children and recovery is achieved only after re-nutrition or treatment of the immunodeficiency (Gendrel *et al.*, 2003). A study of children in Mexico showed a 40% greater risk of cryptosporidiosis among poor urban children (Miller *et al.*, 1994).

The prevalence of *Cryptosporidium* infection in children along the Texas-Mexico border was associated with some risk factors such as lower household income and socioeconomic status, source of water supply and age (Leach *et al.*, 2000).
The seasonal distribution and climate condition are other risk factors in relationship with epidemiology of cryptosporidiosis. The seasonal distribution of cryptosporidiosis varies in different parts of the world (Hart, 1999). 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 fertilizer (Clavel *et al.*, 1996). In Australia, the majority of *Cryptosporidium* spp. infection cases were between January and March (Puech *et al.*, 2001). In tropical countries such as Kuwait the maximum numbers of cases, 38 of 51 (75%), were seen during the months January to April indicating a marked seasonal variation (Iqbal *et al.*, 2001).

Hart (1999) showed that there was no influence of gender in *Cryptosporidium* cases, however two recent reports concluded that in *Cryptosporidium* infections in children with diarrhoea there are differences between the sexes (Huang *et al.*, 1998; Puech *et al.*, 2001).

Hellard *et al.* (2003) concluded that sexual behaviour is a significant risk factor for cryptosporidial diarrhoea in homosexual men, (Hellard *et al.*, 2003).

Diarrhoea in the family and overcrowding were associated with *Cryptosporidium* infection in some studies. For instance, Newman *et al.* (1994) found a high transmission rate of *Cryptosporidium* infections in households with identified persons who had cryptosporidiosis in an urban slum in Fortaleza, Brazil (Newman *et al.*, 1994).

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). A single oocyst is sufficient to produce infection and disease in susceptible hosts (Pereira *et al.*, 2002). A case-control study demonstrated a strong statistical association between illness and the consumption of unboiled tap water from a particular source with evidence of a dose-response relationship (Joseph *et al.*, 1991).

1.12. Immune status and responses

In normal hosts cryptosporidiosis is self-limited, while in immunocompromised hosts such as patients with AIDS, persistent overwhelming diarrhoea frequently develops, (Thompson *et al.*, 2005) with dissemination of parasite throughout the gastrointestinal tract and spread involving the colon, bile ducts, gall bladder, pancreas and associated mucosal surfaces (Hunter & Nichols, 2002). 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). Disseminated cryptosporidiosis can occur in individuals with AIDS. AIDS is present when the CD4 T cell count is 750 cells/mm^3 or less in children younger than 12 months, 500 cells/mm^3 or less in children 1 to 5 years of age, 200 cells/mm^3 or less in children 6 to 12 years of age and 200 cells/mm^3 or less in adults and adolescents (Huang *et al.*, 2004). $CD4^+$ T lymphocytes are necessary for the resolution of diarrhoea and infection, and experiments

have shown that immunity is dependent on the number of $CD4^+$ T cells. These help in increasing the intraepithelial lymphocyte population and generating gamma interferon (Dwivedi *et al.*, 2007). In a recent study to identify various causative agents of diarrhoea among HIV infected individuals and the role of associated risk factors and immune status, *Cryptosporidium* was found to be an important parasite in HIV positive patients with diarrhoea ($P<0.01$). Polyparasitic infection was further observed in diarrhoeal patients (chronic), particularly in those with a lower $CD4^+$ count. Therefore when assessing a patient's immune status and enteric pathogenic profile, it is recommended that a routine screening for faecal specimens is performed and that the $CD4^+$ count is obtained (Dwivedi *et al.*, 2007).

1.13. Transmission

The oocyst is the stage transmitted from an infected host to a susceptible host by the faecal-oral route. Cryptosporidiosis has many biological and epidemiological features that promote transmissibility and which complicate control measures. These include: oocysts that 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 (Casemore, 1990; Fayer *et al.*, 1997).

1.13.1. Zoonotic transmission (animal to human 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.*, 1982). Conversely since many species of animals are known to harbour the parasite infection can be transmitted from peri-domestic animals. Most studies have focused on *C. parvum* which appears to have the broadest host range of the currently recognized species of *Cryptosporidium* (Thompson *et al.*, 2005). Direct transmission of *C. parvum* from animals to humans is well documented

for example, the outbreak of cryptosporidiosis among veterinary students in the USA clearly demonstrates the potential for zoonotic transmission (Current *et al.*, 1983). The importance of zoonotic transmission has been recently stressed by the observation of a dramatic decline (81%) in the incidence of human *C. parvum* cryptosporidiosis in the UK coinciding with a foot and mouth disease outbreak, which occurred in Spring 2001 (Hunter *et al.*, 2003).

A recent investigation has demonstrated that the decrease in spring incidence of infection, (the spring peak is essentially caused by the zoonotic species *C. parvum*), observed in northwest England is closely related to the measures taken by the water suppliers to improve the treatment of drinking water (Sopwith *et al.*, 2005). *C. parvum* from cattle and sheep have been clearly demonstrated to be transmitted to humans but isolates from other animals may not be infective to humans (Sulaiman *et al.*, 1998). Direct contact with animals or contaminated food has also been linked to outbreaks of *C. parvum* cryptosporidiosis in humans in North America and Europe (Stantic-Pavlinic *et al.*, 2003). Dogs and cats appear to be most commonly infected with the predominantly host adapted *C. canis* and *C. felis* and as such dogs, cats and other companion animals may not be important zoonotic reservoirs of *Cryptosporidium* infection (Hunter & Thompson, 2005).

1.13.2. Water-borne transmission and outbreaks

Water presents a major route of transmission, both 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 organizations 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. Waterborne outbreaks of cryptosporidiosis have been documented in many countries worldwide, and over 45 outbreaks have been reported with the greatest documentation in the USA and UK (Fayer, 2004). These may be huge, as in the Milwaukee waterborne outbreak which affected an estimated 403,000 individuals (MacKenzie *et al.*, 1995), with a total cost of outbreak-associated illness of US$96.2 million (Corso *et al.*, 2003). Most outbreaks have implicated contaminated surface water but a significant number were associated with contaminated groundwater e.g. wells and springs not properly protected from sewage and run-off (Caccio & Pozio, 2006).

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 process. *Cryptosporidium* is also one of the most commonly recognized causes of recreational waterborne disease which occurs by feacal contamination or cross connection in swimming pools. Faecal contamination coupled with oocyst resistance to chlorine, low infectious dose and high bather densities facilitate transmission (Fayer, 2004).
Few studies have characterized 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). *C. hominis* was responsible for more outbreaks than *C. parvum* even in countries where the latter is the predominant human parasite such as in the UK (Caccio *et al.*, 2005).

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).

1.13.3. Food borne transmission

The role of food in the epidemiology of cryptosporidiosis has been more difficult to investigate and not well documented. Numerous raw vegetables collected in developing and developed countries were found to be contaminated with oocysts, (Millar *et al.*, 2002). Oocysts of *C. parvum* have been detected in shellfish, (several species of mussels, clams and oysters), although in no case were these associated with outbreaks of cryptosporidiosis in humans (Fayer, 2004; Fayer *et al.*, 2004). Epidemiological evidence from the UK has shown that consumption of specific foods such as offal and raw fresh sausage is associated with *Cryptosporidium* infection (Casemore, 1990). Cryptosporidiosis has also been attributed to ingestion of contaminated apple juice, chicken salad, milk and food prepared by an ill food handler (Millar *et al.*, 2002). Mechanical transmission by cockroaches and flies has been documented (Graczyk *et al.*, 1999).

1.13.4. Person to person transmission

Person to person spread of *Cryptosporidium* is one of the most common modes of transmission. Cases of human-to-human transmission have been reported between family members, sexual partners, children in daycare centres and hospital patients and staff (Caccio & Pozio, 2006). 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. 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).

1.14. Epidemiology in patients with HIV-infections

Human immunodeficiency virus (HIV)/AIDS ranks among the most dreaded diseases afflicting mankind, causing dysfunction of both limbs of the immune system resulting in overwhelming and fatal opportunistic infections (Dwivedi *et al.*, 2007).

Gastrointestinal involvement, primarily in the form of diarrhoea, is a universal problem affecting almost 90% of HIV infected patients in developing countries (Mukhopadhya *et al.*, 1999). *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 immmunocompromised 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). Recently Dwivedi *et al.* (2007) reported a high prevalence rate of *Cryptosporidium* (33.3%), as compared to other studies that reported a prevalence ranging between 3.7-11.8% in India (Mukhopadhya *et al.*, 1999; Prasad *et al.*, 2000; Kumar *et al.*, 2002; Mohandas *et al.*, 2002).

Immunocompromised individuals can be infected with more genetically diverse parasite populations (Widmer *et al.*, 1998). 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.*, 2001a; Gatei *et al.*, 2002a; Xiao *et al.*, 2002a).

1.15. Clinical findings

The period from ingestion of oocysts to the appearance of clinical signs is between 2 and 14 days (Thompson *et al.*, 2005). The most common clinical feature of cryptosporidiosis in immunocompetent and immunodeficient patients is diarrhoea, reported by 92% of patients, and it is this symptom that most often leads to diagnosis. Characteristically the diarrhoea is profuse and watery (cholera-like), may contain mucus but rarely blood and leukocytes, and is often associated with weight loss (Caccio & Pozio, 2006). Diarrhoea can last for 10-14 days in the immunocompetent host and for months to years in immunocompromised persons leading to severe dehydration,

malnutrition, extended hospitalizations and mortality (Thompson *et al.*, 2005). The clinical characteristics of *Cryptosporidium* spp. depends on the age and immune status of the host and parasite strain (Farthing, 2000; Tzipori & Ward, 2002; Ramirez *et al.*, 2004). It is believed that adults and children are equally susceptible to infection but children are more likely to develop moderate to severe clinical signs. The relative maturity of the immune system and history of previous exposure may account for the difference in clinical signs between children and adults (Thompson *et al.*, 2005). Other less common clinical features included nausea and vomiting (51% of patients), abdominal pain in (45% of patients) and low grade (<39°C) fever (36% of patients). Occasionally, non-specific symptoms such as myalgia, weakness, malaise, headache and anorexia are reported (Arrowood, 1997; Pozio *et al.*, 1997). However, it is in the immunocompromised host, due to a variety of causes including HIV infection and AIDS, drug treatment in organ transplantation, cancer chemotherapy, etc. (Farthing, 2000), that the infections are most chronic and debilitating. Respiratory tract infections with *Cryptosporidium* spp. have been associated with chronic coughing, dyspnoea, bronchiolitis and pneumonitis (Arrowood, 1997).

1.16. Diagnosis

In the first years after the discovery of this pathogen in humans, diagnosis of cryptosporidiosis required invasive and time-consuming techniques (Caccio & Pozio, 2006). 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 have been designed to detect oocysts by staining, immunological assays or immunofluorescence and molecular techniques (Arrowood, 1997).

1.16.1 Oocyst staining methods

Cryptosporidium oocysts were first described in a histological section of gastric mucosa using Romanovsky's stain (Tyzzer, 1907). Giemsa was later found to stain oocysts in stool, but this method has low specificity, (Tzipori *et al.*, 1980), as yeasts,

which also stain purple, can cause diagnostic confusion. In 1981, Henrickson and Pohlenz 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 *Cryptosporidium* oocysts in the stool and is used extensively in both clinical and research investigations, as it is simple, effective and reproducible. 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). Fluorescent stains include auramine-phenol (AP), auramine-rhodamine and auramine-carbol-fuchsin are used routinely. These fluorescence techniques are said to be more sensitive but less specific compared with the ZN stain (Moodley *et al.*, 1991; Tortora *et al.*, 1992).

Microscopy and staining methods have suffered from the problem of not distinguishing between different species of *Cryptosporidium,* or in distinguishing this parasite from other faecal components of similar size and shape such as yeast cells, algae, moulds and other debris, thus affecting the sensitivity and specificity (Thompson *et al.*, 2005).

1.16.2. Immunoassay methods

Several immunofluorescent assays and enzyme-linked immunosorbent assay (ELISA) kits have become commercially available and are used increasingly 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). ELISA is a widely used assay based on the detection of *Cryptosporidium* soluble antigens in faecal samples. This method is very easy to perform and is useful for screening large numbers of specimens. However according to the author's experience, its specificity is limited by cross reactions with other antigens of parasitic and nonparasitic origin that

generate false positives. Borderline positives and questionable negatives therefore should be confirmed by another independent assay (Caccio & Pozio, 2006).

Recently immunochromatographical lateral-flow immunoassays (rapid assays) for *Cryptosporidium* have become popular diagnostic tools because they eliminate the need for trained microscopists and costly equipment. These tests are simple card assays that have a reported sensitivity of greater than 97% and specificity of 100% (Caccio & Pozio, 2006).

1.17. Treatment

There have been a large number of studies aimed at developing a satisfactory therapy for human cryptosporidiosis, but these investigations have failed to identify a drug of choice. Several groups may benefit from an effective therapy, particularly patients with HIV/AIDS, transplant recipients, patients undergoing cancer chemotherapy and those with severe malnutrition (Caccio & Pozio, 2006). Over 200 compounds were tested, both *in vitro* and *in vivo* but none of them proved to be fully effective against the parasite (Armson *et al.*, 2003) a few have demonstrated an ability to reduce the magnitude of the symptoms, none eliminates the disease completely (Ramirez *et al.*, 2004). Non-specific supportive treatment, including rehydration and nutritional supplementation remains the mainstay of management of the clinical manifestation of cryptosporidiosis (Tzipori & Ward, 2002). Improving the immune status of HIV positive individuals with highly active antiretroviral therapy (HAART) is the treatment of choice for cryptosporidiosis in AIDS patients (Pozio & Morales, 2005), particularly if protease inhibitors are included in the HAART regimen (Hommer *et al.* 2003).

The unique location of the parasite in the host cell (intracellular but extracytoplasmatic), which may affect drug concentration (transported from the host cell across to the parasite) 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). Despite this, a number of drugs (e.g., paromomycin, azithromycin and nitazoxanide) produce improvements in immunocompetent patients (Armson *et al.*, 2003;

Rossignol *et al.*, 2006). Today the therapy of choice is nitazoxanide, a synthetic agent that has demonstrated activity against a broad range of parasites. Rossignol *et al.* (2006) concluded that a 3-day course of this drug is effective in treating diarrhoea and enteritis caused by *Cryptosporidium* in non immunodeficient patients 12 years of age and older.

Spiramycin, a macrolide antibiotic, produced encouraging results in pilot studies but its efficacy in HIV-positive patients has not been demonstrated in controlled clinical trials (Gentile *et al.*, 1991). Paromomycin, an aminoglycoside antibiotic that is poorly absorbed from the gastrointestinal tract, has been one of the most widely used agents to treat cryptosporidiosis in AIDS patients despite poor evidence for efficacy in controlled trials (Bissuel *et al.*, 1994; Hewitt *et al.*, 2000). .

1.18. Prevention and control

Preventive measures for *Cryptosporidium* are by far the most effective approach to control this parasite and fall into two main strategies, prevention of ingestion of the *Cryptosporidium* oocysts and inducing immunity by passive or active immunization (Hart, 1999). As transmission most commonly takes places through water or directly, drinking water quality and good general hygiene (especially in hospitals) are essential for the prevention of the infection. Control of infection within urban groups (e.g. families and daycare centres) are those appropriate for any diarrhoeal disease (Caccio & Pozio, 2006). Immunocompromised patients, including people with a primary immunodeficiency affecting T cells and children with acute leukaemia should avoid contact with animals with diarrhoea, young pets and should avoid swallowing water while swimming. Furthermore treatment of drinking water to inactivate oocysts, either by boiling or filtration, may be necessary under certain circumstances (Hunter & Nichols, 2002).

Passive immunotherapy using oral bovine serum concentrate improved symptoms and reduced oocyst shedding in calves with experimental cryptosporidiosis (Hunt *et al.*, 2002) but colostrum from cows hyper-immunized with *C. parvum* oocysts achieved

limited success in both human and non-human hosts (Gomez Morales & Pozio, 2002). Although it has been shown that immune murine dams do not provide lacteal immunity to cryptosporidiosis to their pups (Moon *et al.*, 1988), a high molecular weight glycoprotein (>9 × 10^5 kDa) *C. parvum* antigen is recognized by the hyper immune bovine colostrum used to treat cryptosporidiosis has been characterized and could be a potential vaccine candidate (Hart, 1999).

2. CRYPTOSPORIDIOSIS IN SAUDI ARABIA AND NEIGHBOURING COUNTRIES

There are few published reports of studies on cryptosporidiosis in the Middle East. In Saudi Arabia, diarrhoeal disease is an important cause of morbidity in children but the contribution made by *Cryptosporidium* spp. is poorly studied.

2.1 Aims

The aims of the present review are:

(1) To document the prevalence of *Cryptosporidium* spp. in humans and animals in Saudi Arabia and neighbouring countries.

(2) To estimate the disease burden.

(3) To examine the age distribution of the cases.

(4) To determine the seasonality of cryptosporidiosis.

(5) To compare the importance of *Cryptosporidium* spp. as a cause of diarrhoea relative to other enteropathogens.

2.2. Methods:-

Papers were identified for this review from a multilingual MEDLINE search for publications from 1976 to 2006 using the keywords of *Cryptosporidium,* and the names of each of the following countries: Saudi Arabia, Kuwait, Iraq, Jordan, Bahrain, Qatar, Yemen, Oman and United Arab Emirates. In addition, the *Annals of Saudi Medicine* and the *Saudi Medical Journal* were hand searched for papers on diarrhoeal diseases and *Cryptosporidium.*

The studies were grouped into three categories: studies targeting human populations; studies targeting animal populations; and studies identifying *Cryptosporidium* species and typing and were analysed separately.

For each study, the prevalence of *Cryptosporidium* infection was examined initially in patients and compared with controls where a control group was included. Age profile and seasonality were also examined if sufficient data were available.

Finally, we reviewed studies in which molecular characterisation, typed species and subtyping allele families were available to examine the distribution of the different *Cryptosporidium* species and strains in circulation.

2.3. Results

The results are presented in Tables 2.1 to 2.3, and are described in the sections below:

2.3.1. Description of Studies

Using the above keywords in MEDLINE and hand searching revealed a total of 23 papers published from the region between 1986 and 2006. The prevalence of *Cryptosporidium* infection among humans was addressed by 15 papers (Khan *et al.*, 1988; Hira *et al.*, 1989; Daoud *et al.*, 1990; Bolbol, 1992; Nimri & Batchoun, 1994; Nimri & Hijazi, 1994; Youssef *et al.*, 2000; Iqbal *et al.*, 2001; Mahdi & Ali, 2002b; Mahdi & Ali, 2002a; Al-Braiken *et al.*, 2003; Nimri, 2003; Mahdi & Ali, 2004; Mahgoub *et al.*, 2004; Nimri & Meqdam, 2004), while the prevalence among animals was addressed by two papers (Mahdi & Ali, 2002a; Al-Atiya *et al.*, 2004). In addition, 5 papers identified the circulating species and reported the molecular characterisation of the parasite (Mahdi & Ali, 1999; Abo-Shehada *et al.*, 2004; Mahgoub *et al.*, 2004; El-Mathal & Fouad, 2005; Sulaiman *et al.*, 2005). One paper reported an outbreak of caprine cryptosporidiosis in the Sultanate of Oman and another examined the prophylactic value of paromomycin in the same outbreak (Johnson *et al.*, 1999; Johnson *et al.*, 2000). Finally, one paper described a case of Cryptosporidiosis in a child in Kuwait (Britt & Al-Ghawaby, 1988).The studies included five from Saudi Arabia (Khan *et al.*, 1988; Bolbol, 1992; Al-Braiken *et al.*, 2003; Al-Atiya *et al.*, 2004; El-Mathal & Fouad, 2005) and the remainder from four neighbouring countries namely; Kuwait (Britt & Al-Ghawaby, 1988; Hira *et al.*, 1989; Daoud *et al.*, 1990; Iqbal *et al.*, 2001; Sulaiman *et al.*, 2005), Jordan (Nimri & Batchoun, 1994; Nimri & Hijazi, 1994; Youssef *et al.*, 2000; Nimri, 2003; Abo-Shehada *et al.*, 2004; Mahgoub *et al.*, 2004; Nimri & Meqdam, 2004), Iraq (Mahdi & Ali, 1999; Mahdi & Ali, 2002b; Mahdi & Ali, 2002a; Mahdi & Ali, 2004) and The Sultanate

of Oman (Johnson *et al.*, 1999; Johnson *et al.*, 2000).Ten of these studies were hospital-based (Britt & Al-Ghawaby, 1988; Hira *et al.*, 1989; Daoud *et al.*, 1990; Bolbol, 1992; Mahdi & Ali, 1999; Youssef *et al.*, 2000; Mahdi & Ali, 2002b; Mahdi & Ali, 2004; Mahgoub *et al.*, 2004; Sulaiman *et al.*, 2005), four studies were designed to select inpatient cases only (Britt & Al-Ghawaby, 1988; Daoud *et al.*, 1990; Youssef *et al.*, 2000; Mahdi & Ali, 2002b), whereas both inpatients and outpatients were included in another four studies (Hira *et al.*, 1989; Bolbol, 1992; Mahdi & Ali, 2004; Mahgoub *et al.*, 2004) and seven studies targeted hospital outpatients only (Khan *et al.*, 1988; Nimri & Hijazi, 1994; Mahdi & Ali, 1999; Iqbal *et al.*, 2001; Al-Braiken *et al.*, 2003; Nimri & Meqdam, 2004; El-Mathal & Fouad, 2005) . In addition seven studies were entirely community based (Nimri & Batchoun, 1994; Johnson *et al.*, 1999; Johnson *et al.*, 2000; Mahdi & Ali, 2002a; Nimri, 2003; Abo-Shehada *et al.*, 2004; Al-Atiya *et al.*, 2004) (Table3.1).

2.3.2. Detection methods

In most studies faecal specimens were screened by examining stained faecal smears by bright field microscopy and the most common stains used were acid fast stains, (16 studies) (Khan *et al.*, 1988; Nimri & Batchoun, 1994; Nimri & Hijazi, 1994; Johnson *et al.*, 1999; Mahdi & Ali, 1999; Johnson *et al.*, 2000; Mahdi & Ali, 2002b; Mahdi & Ali, 2002a; Al-Braiken *et al.*, 2003; Nimri, 2003; Abo-Shehada *et al.*, 2004; Al-Atiya *et al.*, 2004; Mahdi & Ali, 2004; Mahgoub *et al.*, 2004; Nimri & Meqdam, 2004; El-Mathal & Fouad, 2005) followed by trichrome, (7 studies) (Hira *et al.*, 1989; Daoud *et al.*, 1990; Nimri & Batchoun, 1994; Nimri & Hijazi, 1994; Youssef *et al.*, 2000; Nimri, 2003; Nimri & Meqdam, 2004) and safranin methylene blue, (5 studies) (Britt & Al-Ghawaby, 1988; Hira *et al.*, 1989; Daoud *et al.*, 1990; Bolbol, 1992; Iqbal *et al.*, 2001). Fluorescence microscopy was used to examine faecal smears stained with auramine-phenol, (4 studies) (Khan *et al.*, 1988; Johnson *et al.*, 1999; Johnson *et al.*, 2000; Youssef *et al.*, 2000). Other techniques such as a direct immunofluorescence assay (DFA) were used in three studies (Iqbal *et al.*, 2001; Abo-Shehada *et al.*, 2004; Mahgoub *et al.*, 2004) while polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) were used in one study from Kuwait (Sulaiman *et al.*, 2005). Different techniques used prior to

microscopy include sedimentation, sugar flotation concentration and direct wet mount preparations.

2.3.3. Duration of the studies

Study duration was variable, ranging from 3 months (Hira *et al.*, 1989; Al-Braiken *et al.*, 2003) to 38 months (Sulaiman *et al.*, 2005).The median study duration was 12 months.

2.3.4. Ages of the patients

The ages of the patients surveyed varied significantly, ranging from newborn (0-1 month) (Bolbol, 1992) to 87 years (Nimri, 2003) and even within the same study the age groups ranged from 1 to 87 years (Nimri, 2003).

2.3.5. Ages of the animals

Animal ages were reported in only the study from The Sultanate of Oman (Johnson *et al.*, 1999) and they ranged between two days to over one year.

2.3.6. Prevalence

In humans the prevalence of cryptosporidiosis ranged from 1% (Khan *et al.*, 1988) to 37.3% (Mahgoub *et al.*, 2004) and seemed to be higher in the studies that targeted infants and children under 7 years old (Table 2.1). In animals, it was different for different species of animals and the geographic locations of the studies (Table 2.2). The prevalence of Cryptosporidiosis differed depending on the population surveyed. Outpatient studies showed significantly higher prevalences than those where inpatients were included. In addition, mixed inpatient and outpatient studies showed higher prevalences than the community-based studies. The range and median in outpatient prevalence studies were 1 to 32% and 6.7% respectively, whereas the range and median of combined inpatient and outpatient studies were 1.15 to 37.3% and 5.5% respectively.

Cryptosporidium infection was more common in children less than 7 years of age. Data from 8 studies showed that the prevalence of cryptosporidiosis was higher in

children less than 7 years old (Khan *et al.*, 1988; Bolbol, 1992; Nimri & Hijazi, 1994; Youssef *et al.*, 2000; Iqbal *et al.*, 2001; Mahdi & Ali, 2002b; Mahdi & Ali, 2002a; Al-Braiken *et al.*, 2003; Mahgoub *et al.*, 2004), and 4 of these studies showed that the highest prevalence was among children less than 2 years of age (Khan *et al.*, 1988; Youssef *et al.*, 2000; Iqbal *et al.*, 2001; Al-Braiken *et al.*, 2003).

2.3.7. Seasonality

The seasonal variation in cryptosporidiosis was reported in 5 studies only. Two studies from Kuwait, which has high temperatures during the summer with a very dry climate and a short cool winter, showed that the highest prevalence was during winter, i.e. March and April (Daoud *et al.*, 1990), or from January to April (Iqbal *et al.*, 2001). The other 3 studies were from Jordan which has a relatively wet rainy season from November to April and very dry weather for the rest of the year with uniformly hot dry summers and cool variable winters. The maximum prevalence in the first study which was done in Irbid City was undertaken from January to May in the rainy season (Mahgoub *et al.*, 2004), while in the two remaining studies, which were done in Badia, the maximum prevalence was reported in the warm months from May to September (Nimri, 2003; Nimri & Meqdam, 2004).

2.3.8. *Cryptosporidium* species & strains identified

The most commonly identified species was described as *Cryptosporidium parvum* which has been reported from Dammam, Saudi Arabia (El-Mathal & Fouad, 2005), Kuwait (Sulaiman *et al.*, 2005), Irbid, Jordan (Mahgoub *et al.*, 2004), Basra, Iraq (Mahdi & Ali, 1999) and Bani-Kenanah in Jordan,(Abo-Shehada *et al.*, 2004) (Table2.3). *Cryptosporidium hominis* was reported in one study from Kuwait only (Sulaiman *et al.*, 2005). Light and immunofluorescent microscopy were used to identify *Cryptosporidium* species in these studies except in the study from Kuwait where advanced molecular tools were used namely PCR (Polymerase Chain Reaction), RFLP (Restriction Fragment Length Polymorphism) and DNA sequencing. It is not possible to assign cryptosporidia to species on the basis of microscopic morphology alone.

In the last study (Sulaiman *et al.*, 2005) 4 subtype allele families (IIa, IId, IIc &IIf) of *Cryptosporidium parvum* were identified and 3 (Id, Ie & Ib) of *Cryptosporidium hominis.* An atypical outbreak of caprine cryptosporidiosis occurred in the Sultanate of Oman (Johnson *et al.*, 1999). It occurred in goats ranging in age from two days to adulthood, on a well-managed closed farm. None of the other animals on the farm, including sheep, cows and buffalo were affected. Morbidity approached 100 per cent in goats less than six months of age. Despite intensive supportive care 238 goats died ranging in age from two days to over one year. Cryptosporidia were detected in large numbers in the intestinal contents of dead animals and in faecal smears of animals with diarrhoea.

In another report from the Sultanate of Oman, the prophylactic value of paromomycin, an aminoglycoside antibiotic analogous in structure to neomycin, (Schillings & Schaffner, 1961) was examined in the same outbreak of caprine cryptosporidiosis (Johnson *et al.*, 2000).

Table 2.1. *Cryptosporidium* detection rates in Saudi Arabia and neighbouring countries (from Areeshi *et al.*, 2007).

Country	Period	Detection methods	Number of patients	Age of patients	Number of control	Age	Setting	Prevalence in patients	Prevalence in control	Seasonality	Reference
Saudi Arabia	Mar2000 to May2000	S, DWM& BFM(AFS)	63	17 %<1 year	190	<5 years	(O)	32 %	4.7 %	NR	(Al-Braiken *et al.*, 2003)
Saudi Arabia	1990	S&BFM(SM-B)	174	25-59 months	50	0-120 months	H(I&O)	1.15 %	0 %	NR	(Bolbol, 1992)
Saudi Arabia	Nov 86 to May 87	DWM, S , BFM (AFS)& FM (AP)	209 *	2 years	0	0-12 years	(O)	1 %	0 %	NR	(Khan *et al.*, 1988)
			112 **			>12years		0 %			
Kuwait	Jan1988 to Jun 1999	DWM, S , BFM(SM-B)& TSSP	2205	5-96 months	0	2 weeks to 12 years	H(I)	1.6 %	0	Winter March & April	(Daoud *et al.*, 1990)
Kuwait	mid-Jan to mid Apr1989	S , DWM, BFM(SM-B)& TSSP	738 *	1.25-8 years	0	NR	H(I&O)	1.36 %	0 %	NR	(Hira *et al.*, 1989)
			413 **					0 %			
Kuwait	Sep1995 to Aug1997	S, BFM(SM-B) & DFA	3549	>2 years	500	3 months to 13 years	(O)	1.43 %	0 %	Winter Jan to Apr	(Iqbal *et al.*, 2001)
Jordan	Nov 2000 to Sep 2001	DWM, SFC, BFM(AFS) & DFA	300	5-7 years	0	0 to 12 years	H(I&O)	37.3 %	0 %	Jan to May, the rainy season	(Mahgoub *et al.*, 2004)
Jordan	Sep1992 to Mar 1993	DWM, S, SFC, & BFM(AFS,TSSP)	1000	<9 years	0	6 to 14 years	CB	4 %	0 %	NR	(Nimri & Batchoun, 1994)
Jordan	Sep1999 to Sep2001	DWM, S, SFC, BFM(AFS,TSSP)	200	Mean 7.5 years	0	1 to 14 years	CB	5 %	0 %	Spring	(Nimri, 2003)
						15 to 87 years		3 %			
Jordan	NR	DWM, S, BFM(AFS,TSSP)	180	NR	100	12 – 84 years	(O)	8.3 %	0 %	Warm months May-Sep	(Nimri & Meqdam, 2004)
Jordan	May to Aug 1993	S, BFM(TSSP) & FM(AP)	265	<1 year	0	< 5years	H(I)	1.5 %	0 %	NR	(Youssef *et al.*, 2000)
Iraq	Nov 1997 to May 1998	S, BFM(AFS)	40	46- 55 years	175	2 years - 60 years	H(I)	5 %	1.14 %	NR	(Mahdi & Ali, 2002b)
Iraq	NR	S, BFM(AFS)	60	<6 years	175	4-62 years	CB	5 %	1.14 %	NR	(Mahdi & Ali, 2002a)
Iraq	Jan to Dec2000	S, BFM(AFS)	205	26-35 years	175	2 months- 65 years	H(I&O)	9.7%	1.1%	NR	(Mahdi & Ali, 2004)
Jordan	Jul 1992 to Sep 1993	DWM, SFC, BFM(TSSP,AFS)	300	<3 years	300	6 months-6years	(O)	6.7%	1.7%	NR	(Nimri & Hijazi, 1994)

S: Sedimentation DWM: Direct Wet Mount BFM: Bright Field Microscope FM: Fluorescent Microscope DFA: Direct Immunofluorescent Assay SFC: Sugar Flotation Concentration
NR: not reported. Hb: Hospital based I: Inpatient O: Outpatient Cb: community based * children ** adult SM-B: Safranin Methylene Blue
AP: Auramin-phenol TSSP: Trichrom Stool Smear Preparation AFS: Acid Fast Stain(modified Ziehl-Neelsen & modified kinyouns)

Table 2.2. *Cryptosporidium* detection rates among animals in Saudi Arabia and neighbouring countries (from Areeshi *et al.*, 2007).

Country	City	Period	Duration	Animal type	Detection methods	Number of animals	Prevalence	Reference
Saudi Arabia	Al-Ahsa region	Oct 2002 to Oct 2003	13 months	White-cheeked bulbuls	BFM(AFS)	42	28.6 %	(Al-Atiya *et al.*, 2004)
Iraq	Basrah	NR	NR	Domestic animals	S, BFM(AFS)	198	13.6 %	(Mahdi & Ali, 2002a)

Nr: not reported BFM: Bright Field Microscope AFS: Acid Fast Stain(modified Ziehl-Neelsen & modified Kinyouns)
S: Sedimentation

Table 2.3. *Cryptosporidium* species identified and subtyping (from Areeshi *et al.*, 2007).

Country	City	Species identified	%	Subtyping	Technique	Reference
Saudi Arabia	Dammam	*C. parvum*	30	-	S,BFM(E&I, AFS)	(El-Mathal & Fouad, 2005)
Kuwait	Kuwait	*C. parvum*	94	4 subtype allele families (IIa, IId. IIc & IIf).	SSU rRNA-based PCR-RFLP & 60-KD a glycoprotein-based DNA sequencing tool.	(Sulaiman *et al.*, 2005)
		C. hominis	5	3 subtype allele families (Id, Ie & Ib).		
Jordan	Irbid	*C. parvum*	37.3	-	DWM,SFC, BFM(AFS) & DFA	(Mahgoub *et al.*, 2004)
Iraq	Basra	*C. parvum*	1		S, BFM(AFS)	(Mahdi & Ali, 1999)
Jordan	Bani-Kenanah	*C. parvum*	2	-	BFM(AFS), DFA	(Abo-Shehada *et al.*, 2004)

DFA: Direct Immunoflourescent Assay BFM: Bright Field Microscope RFLP: Restriction Fragment Length Polymorphism E&I: Eosin & Iodine stain

2.4. Discussion

Diarrhoea remains a major cause of morbidity and mortality worldwide particularly in developing countries. In this review a total of 23 studies addressing *Cryptosporidium* infections in Saudi Arabia and neighbouring countries have been reviewed in terms of prevalence, age distribution, seasonality, and molecular characterization. Significant differences were observed in the prevalence of *Cryptosporidium* infection in humans which ranged between 1% (Khan *et al.*, 1988) and 37.3% (Mahgoub *et al.*, 2004), while in animals it varied for different species and the geographic locations of studies. It appears that the prevalence of cryptosporidiosis in humans is lower than in animals (Mahdi &Ali, 2002a). Interestingly, the prevalence differed even within Irbid, a city in Jordan, where the prevalence varied from 1.5% (Youssef *et al.*, 2000) to 37.3% (Mahgoub *et al.*, 2004). This may be due to methodological differences, as bright field microscopy only was used in most of the studies with its low detection rate compared to DFA which is more sensitive. For example, in Irbid the detection rate was 37.3% in the study where DFA was used together with bright field microscopy (Mahgoub *et al.*, 2004), while in an other study in the same city where bright field microscopy alone was used , the detection rate decreased to 1.5% (Youssef *et al.*, 2000).

The finding that most *Cryptosporidium* infections occur among children less than 7 years of age is consistent in with most studies in the region and is comparable with reports from other parts of the world (Tumwine *et al.*, 2003, Steinberg *et al.*, 2004, Oshiro *et al.*, 2000).

It is possible that the infection rate in these studies would have been higher if more than one stool specimen had been collected from each child because of the intermittent nature of oocyst excretion of this parasite (Navin &Juranek, 1984, Tzipori *et al.*, 1983). Outpatient studies showed significantly higher prevalences than those where inpatients were included which may have resulted from the presence of *Cryptosporidium* oocysts in asymptomatic children, some of whom could be considered carriers who act as important reservoirs of the organism and finally a potential source of infection. Seasonal variations in prevalence have been noted in some studies (Daoud *et al.*, 1990). Several

factors could account for seasonal variations in the occurrence of cryptosporidiosis, including factors affecting the numbers 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. However in most studies, the highest numbers of cases were detected during the rainy season (Javier Enriquez *et al.*, 1997, Katsumata *et al.*, 1998). In Kuwait for example the climate is characterized by long dry hot summers (40-50 °C) and short warm winters. There is no rainy season in Kuwait as such. The highest prevalence was during winter (March and April) (Daoud *et al.*, 1990) or from January to April (Iqbal *et al.*, 2001).While in Jordan, which has a relatively moist rainy season from November to April and very dry weather for the rest of the year with hot dry uniform summers and cool variable winters, the maximum prevalence was from January to May, The rainy season, in Irbid (Mahgoub *et al.*, 2004) but in the warm months from May to September in Badia (Nimri, 2003, Nimri &Meqdam, 2004).

Few studies have tried to identify the infecting species and *Cryptosporidium parvum* was the most prevalent species identified but this was with one exception by microscopy alone. *Cryptosporidium hominis* was identified in a study from Kuwait (Sulaiman *et al.*, 2005) where RFLP was used. Because the majority of these studies were done between, 1986 and1998, no advanced molecular tools were used to identify the infecting species and the researchers depended completely upon microscopy and Direct Immunofluorescent Assay as a screening tool which calls into question the accuracy of the results. In the one study where molecular tools such as PCR and RFLP were used *C. hominis* (5% of cases) and *C.parvum* (95%) were detected (Sulaiman *et al.*, 2005).

The distribution of *Cryptosporidium* genotypes in the population is very different world wide. Studies conducted in Peru, Thailand, Malawi, Uganda, Kenya, and South Africa showed a predominance of *C. hominis* in children or human immunodeficiency virus- positive adults (Gatei *et al.*, 2003, Leav *et al.*, 2002, Peng *et al.*, 2003a, Tiangtip &Jongwutiwes, 2002, Xiao *et al.*, 2001). In contrast, most of the patients investigated in Kuwait were infected with *C. parvum.* The only other region where *C. parvum* is more prevalent in humans than *C. hominis* is Europe, where most studies have shown a slightly

higher prevalence of *C. parvum* over *C. hominis* in both immunocompetent and immunocompromised persons (Alves *et al.*, 2003, Chalmers *et al.*, 2002, Guyot *et al.*, 2001, McLauchlin *et al.*, 2000).

The differences in the distribution of *Cryptosporidium* genotypes in humans are considered an indication of differences in infection sources (Learmonth *et al.*, 2001, Learmonth *et al.*, 2004, McLauchlin *et al.*, 2000). Thus a predominance of *C. parvum* in a population has been considered to be the result of zoonotic transmission. Indeed, even in areas with a high percentage of infections due to *C. parvum*, massive slaughter of farm animals during foot-and-mouth disease outbreaks can result in a reduction of the proportion of human infections due to *C. parvum* (Smerdon *et al.*, 2003).

Recent subtyping studies have shown that not all *C. parvum* infections in humans are the result of zoonotic transmission (Alves *et al.*, 2003, Mallon *et al.*, 2003, Xiao *et al.*, 2004). Among the *C. parvum* GP60 subtype families identified, alleles IIa and IIc (previously known as Ic) are the two most common types. The former has been identified in both humans and ruminants, whereas the latter has been seen only in humans (Alves *et al.*, 2003, Peng *et al.*, 2003b, Xiao *et al.*, 2004). In one of the studies included in this review two Kuwaiti children were infected with an allele IIc subtype strain, indicating that anthroponotic transmission of *C. parvum* occurs in Kuwait. Nevertheless, the low proportion of infections due to *C. hominis* suggests that anthroponotic transmission of cryptosporidiosis in Kuwait is probably not as important as in other countries. It is important to note that none of the other valid taxonomic species have yet been detected in the region.

Returning to the outbreak of caprine cryptosporidiosis in the Sultanate of Oman, and to determine the reasons for it, it has been noted that the outbreak started during the cooler rainy season and this environmental factor might have imposed a stress responsible for the epizootic. However the rainy season may be associated with other factors, such as the enhanced survival of the organism in the environment or the seasonal variability of antibody levels observed in goats. Johnson *et al.* (Johnson, 1995) observed an increase in incidence of enteric and respiratory diseases in goats during the rainy season in north-east Brazil and associated it with lower levels of serum antibodies

recorded during the wet season than during the dry hot season. It has also been reported that goats who do not receive colostrum or are separated from their mothers and fed artificially, are more likely to develop cryptosporidial infections (Tzipori *et al.*, 1981, Card *et al.*, 1987, Vieira *et al.*, 1997). The kids in the Omani study were all suckled by their mothers and there was no indication that they had not received adequate quantities of colostrum. No immediate source of the infection was apparent. The water supply was not tested for cryptosporidia but it seemed unlikely to have been the source because no other animals were affected and the treated water supply for the animals was the same as that for human consumption. It was a closed herd and the possibility that the infection was brought in from outside could therefore be ruled out.

2.5. Conclusions:-

Based on this review we put forward the following recommendations for future studies.

Future studies should focus predominantly on those in the age group <7 years, as this is consistent with the findings of previous studies in the region and elsewhere. The period of the study should be at least 12 months in order to be able to detect any seasonal variation. Wherever possible, molecular techniques should be used for typing *Cryptosporidium* in order to provide valuable information about all circulating *Cryptosporidium* species and strains in the region. Finally we recommend that physicians be aware of *Cryptosporidium* as a cause of diarrhoea in children and that procedures for the diagnosis of this parasite is included in the routine diagnostic procedure for diarrhoeal stool specimens in all laboratories. This is particularly important since there is now an effective drug, nitazoxanide available for therapy (Rossignol *et al.*, 2006).

2.6. Objectives

There are very few published reports on the prevalence of various genotypes of *Cryptosporidium* in humans and animals in Saudi Arabia. The present study is the first reporting *Cryptosporidium* prevalence in two major areas in Saudi Arabia, Gizan situated in the southwest and Madina in the northwest.

The main objectives of the study were to determine the following:

1- The relative importance of *Cryptosporidium* as a cause of childhood diarrhoea among other enteropathogens in Saudi Arabia.

2- The distribution and genotypes of different *Cryptosporidium* species in Saudi Arabia.

3- The age specific prevalence of cryptosporidiosis in Saudi Arabia.

4- The relationship between *Cryptosporidium* in humans and domestic animals in the south west of Saudi Arabia.

5- The sensitivity and specificity of routine laboratory and molecular diagnostic methods for the diagnosis of cryptosporidiosis.

6- To examine possible differences in the prevalence, age distribution and circulating genotypes of *Cryptosporidium* in Gizan and Maddina, Saudi Arabia.

3. MATERIALS AND METHODS

3.1 Sample collection and sources

Two different areas in Saudi Arabia were chosen for this study, Gizan situated in the south west of the kingdom and Maddina situated in the north west of the kingdom (Figure3.1). The climate in Gizan which is an important port on the Red Sea (Figure 3.2) is hot and wet during the summer months, (April to October) and warm during the winter months, (November to March), while in Maddina it is hot and dry for most of the year extending from April to October, followed by a cool, dry season between December to February. A total of 1641 human samples were obtained from the two areas, 516 and 1125 from Gizan and Maddina respectively. In addition, 279 samples were collected from young animals in the Gizan area.

The majority of the human samples from Gizan were collected from children aged between 0 months to 6 years visiting a hospital or a primary health care centre as an inpatient or outpatient, and suffering from gastroenteritis. However some samples were also collected from older patients with gastroenteritis.

Samples were collected from Gizan the capital city (106 samples), and from four other surrounding small cities, Abu Areesh (64 samples), Samtah (106 samples), Al- Ardah (133 samples) and Al-Khobah (45 samples) (Figure 3.3). All the Gizan samples were obtained from the general hospital in each area, except Al-Khobah where samples were obtained from the primary health care centre.

In Maddina, faecal specimens were collected from children under five years of age with acute, dehydrating diarrhoea who were either admitted to the hospitals as inpatients, or who were given oral rehydration therapy (ORT) and treated as outpatients. Maddina samples were obtained from two governmental hospitals. These samples were collected mainly for screening of rotavirus and have been screened for *Cryptosporidium* spp. as well.

A total of 62 samples were collected from asymptomatic school children in year one (5 years age) from Al-Or Elementary school, (29 samples) in the Abu Areesh area and Sager al Jazeera Elementary school, (33 samples) in the Ahad al Masareha area in Gizan (table 3.1).

Figure 3.1. Map of Saudi Arabia. The arrows show the study area locations. Source: The World Factbook (http://www.saudiusrelations.org/)

The children were healthy, normal children and this group was used as the comparison group. The reason for choosing the two schools was that these two areas are centres for animal breeding.

The studies were approved by the Research Ethics Committees, General Directory of Health Affairs in Gizan and Maddina, Ministry of Health, Kingdom of Saudi Arabia (Appendix1-A, 1-B &1C).

Figure 3.2. Map of Gizan, Saudi Arabia. Source: General Directory of Health Affairs in Gizan, Ministry of Health, Kingdom of Saudi Arabia.

Figure 3.3. A picture from Al-Khobah Area, one of the study areas in Gizan.

3.1.1 Sampling methods

3.1.1.1. Hospital sampling

Faecal samples were obtained from patients, usually children, attending medical facilities with symptoms suggestion of gastroenteritis. Patients were recruited from hospitals and clinics in Gizan between October 2004 and February 2005, while in Maddina the collection was done between April 2004 and April 2005. The reasons for the short sample collection period in Gizan were the shortage of laboratory technicians in the hospitals and the long distances among the hospitals, which made the transfer of samples to the deep freezers in Gizan Health Sciences College more difficult. In Maddina the situation was different because the hospitals were very near to the Medical Report Centre in Maddina where the samples were stored.

A formal letter from the General Health Directorate in Gizan was obtained to ask four general hospitals namely: Gizan General Hospital, Abu Aareesh General Hospital, Samtah General Hospital and Al-Ardah General Hospital in addition to Al-Khobah Primary Health Care Centre to participate and help in collecting the stool samples. They were requested to complete a questionnaire (Appendix1-D) stating the serial number of the sample, age and sex of the patient, date of sample collection, whether the patient was treated as an in-patient or an out-patient and the diagnosis of the disease when available.

All the hospitals were selected to cover different regions in the area. A further letter was obtained from General Health Directorate in the Madina area to ask Madina hospitals to participate in the study. All the specimens were stored at -20° C in each hospital, these specimens were transferred in ice blocks to the Medical Report Centre in Maddina or Gizan Health Sciences College Laboratories and stored at -80°C until they were shipped to the Department of Medical Microbiology (DMM&GUM), University of Liverpool, for further analysis.

All samples were kept without preservative with the exception of the animal samples which were preserved in 10% formalin so that they could be imported safely into the UK.

3.1.1.2. School sampling

In order to obtain samples from general population, faeces were collected from school children in Gizan in February 2005. A formal letter from the General Directorate of Education in Gizan was obtained to visit two different Elementary Schools, Al-Or Elementary School in the Abu Areesh area and Sager al Jazeera Elementary School in the Ahad al Masareha area.

Students in year one (5 years of age) in each school were asked to participate in this study. They were all healthy male students. The total number of the year one students was 61 and 67 in Al-Or and Sager al Jazeera Elementary school respectively. The purpose of the study was explained to the principal and the teachers at each school. The parent's information sheet was circulated by the teachers and/or the administration of the schools to the all children's parents in year one in both schools (Appendix1-E). Stool samples were collected from children whom their parents wish them to be included. Attending personally, the samples were kept in labelled clean containers and transferred to deep-freezers in Gizan Health Sciences College Laboratories using freezer bags inside cool boxes to maintain the correct temperature.

3.1.1.3. Animal sampling

Stool collection took place between October 2004 and February 2005. Five large animal farms were visited; three were in the Samtah area. Samples taken included 64 from cows, 57 from goats and 64 from sheep. The two remaining farms were in the Abu Areesh area, samples included 62 from sheep and 32 from cows. The farms were chosen randomly and the total number of animals in each farm ranged from 500 to 600.

The majority of samples were collected from animals less than two years old suffering from diarrhoea and this symptom was the criteria for their inclusion in this study. The samples were collected from the fresh droppings of animals. The samples were kept in labelled clean containers and transferred to deep freezers at Gizan Health Sciences College Laboratories using frozen packs (previously frozen to -80 °C) to maintain the required temperature.

Table 3.1. Sources and numbers of samples examined in the study

Source	Number	Patients	Diarrhoeic
Human samples from Gizan area			
Al-Ardah	133	Children	Y
Al-Khobah	45	Children + Adults	Y
Samtah	106	Children +Adults	Y
Abu Areesh	64	Children	Y
Gizan City	106	Children +Adults	Y
Madina human samples	1125	Children	Y
School collection (comparison group) from Gizan area			
Al-Or Elementary school	29	Children	N
Sager al Jazeera Elementary school	33	Children	N
Animal samples from Gizan area			
Samtah	64	Cows	Y
Samtah	57	Goats	Y
Samtah	64	Sheep	Y
Abu Areesh	62	Sheep	Y
Abu Areesh	32	Cows	Y

Y=Yes N=No

3. 2. *Cryptosporidium* screening

Out of total 1920 samples of both Gizan and Maddina, 795 (all the Gizan samples) were screened by microscopy using a modified Ziehl-Neelsen (ZN) staining technique. Of these, the entire human samples from Gizan (516 (65%)) were screened using Safranin Methylene Blue (SM-B), in addition to ZN. All the 1920 samples in the study were screened by an Enzyme Immunoassay (EIA/ELISA) for detection of *Cryptosporidium* Specific Antigen (CSA).

3.2.1. Ziehl-Neelsen staining method

Equipment and reagents

Frosted glass slides	BDH, UK
Cover slips	BDH, UK
100% methanol	BDH, UK
Carbol fuchsin solution	BDH, UK
Sterile distilled water	
Methylene blue solution	BDH, UK
Immersion oil	BDH, UK
HCl (1N)	Sigma Chemicals, UK
DPX	BDH, UK
Bunsen burner	
Microscope BH-2	Olympus, Japan

Procedure

The method used was as described previously by Casemore *et al.* (1985) and Morgan *et al.* (1998) and was applied with minor modifications. A loopful of the sample was diluted (if necessary) using a loopful of sterile distilled water smeared onto a frosted microscopic glass slide and allowed to air dry for a minimum of 1 hour. The smear was then passed once through a Bunsen flame and then fixed in 100% methanol for 5 minutes and dried. The slide was stained with carbol fuchsin solution for 20 minutes. The smear was then rinsed in tap water for 4 minutes and treated with 1% acid alcohol (1% HCl in 100% methanol for 30

seconds), changing the solution as necessary until fully decolourised and then finally rinsed in tap water for 2 minutes. The slide was then counterstained with 1% methylene blue for 30 seconds, rinsed in tap water for 2 minutes and dried. The slide was then mounted with a coverslip using DPX and scanned at x400 and confirmed by using oil immersion microscopy at x1000 magnification. Each slide was screened for not less than 10 minutes before a negative result is obtained. *Cryptosporidium* oocysts appeared as red oval/round bodies, about 4-5 μm, irregularly stained often with darker staining around the periphery.

3.2.2. Safranin Methylene Blue staining method

Equipment and reagents

Frosted glass slides	BDH, UK
Cover slips	BDH, UK
100% methanol	BDH, UK
Sterile distilled water	
Methylene blue solution	BDH, UK
Safranin	BDH,UK
Immersion oil	BDH, UK
HCL (1N)	Sigma Chemicals, UK
DPX	BDH, UK
Bunsen burner	
Microscope BH-2	Olympus, Japan

Procedure

The method was previously described by Baxby *et al.* (1984). A loopful of the sample was diluted (if necessary) using a loopful of sterile distilled water smeared onto a frosted microscopic glass slid and allowed to air dry for a minimum of 1 hour. The smear was then passed once through a Bunsen flame and then fixed in 3% HCl in 100% methanol for 4 minutes. The slide was washed and flooded with 1% safranin and heated for a total time of two minutes during which the stain boiled once. The slide was washed and counterstained with 1% methylene blue and allowed to air dry. The slide was then mounted with a coverslip

using DPX and scanned at x400 and confirmed by using oil immersion microscopy at x1000 magnification. Each slide was screened for not less than 10 minutes before a negative result is obtained. Oocysts stain a vivid orange-pink and are easily recognised. Electron microscopy was done by Brian Getty on few samples which were detected as very weak positive by ELISA (O.D ranged between 0.06 to 0.08) and they were also negative by this method.

3.2.3. Antigen-EIA/ ELISA technique

To confirm the positivity or the negativity of *Cryptosporidium* cases and to enhance the success of finding more positive cases, an ELISA test was performed on all the samples from cases (n=1858) and comparison group (n=62) 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. Stool specimens were prepared by adding 1 ml Specimen Dilution Buffer (SDB) to a tube and using either one cotton swab coated with unpreserved solid specimen or by adding 0.3ml of preserved or watery unpreserved specimen. Then, 0.2ml of each specimen was added to a well in the assay plate by using transfer pipettes on which rabbit anti-CSA (*Cryptosporidium* Specific Antigen) antibody was bound. If CSA was present, it was captured by the bound antibody. The wells were incubated at room temperature (20-25°C) for 1 hour and then washed to remove unbound material. Four drops (200µl) of the enzyme conjugate (monoclonal anti-CSA antibody labelled with horseradish peroxidase enzyme) was added. The wells were incubated at room temperature (20-25°C) for 30 minutes and then washed to remove unbound conjugate. In a positive reaction, the enzyme conjugate binds to the captured CSA. The substrate for the enzyme, Tetramethyl-Benzadine Buffer (TMB) was added. In a positive reaction the enzyme bound to the CSA converts the substrate to a coloured reaction product. Colour development can be detected visually or spectrophotometrically.

3.2.3.1. Reading of the results

The results of the tests were read within 10 minutes of adding the stop solution (1.0 N sulphuric acid, provided in the kit), both visually and also with a microplate spectrophotometer set at 450 nm. The test was considered valid if the negative control was

colourless or its 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 were considered 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 was less than 0.300, the test should be repeated. The OD of the positive reaction should be ≥0.050 after the OD of the negative control has been subtracted. On the other hand the OD of a 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 colourless.

3.3. Genotyping study

All 104 samples from Gizan and Maddina in Saudi Arabia that were identified as positive by either microscopy or ELISA, were examined at three genetic loci using DNA extracted from whole faeces.

3.3.1. *Cryptosporidium* genomic DNA extraction

Where samples were preserved in 10% formalin (animal samples), the preservative was washed off thoroughly by centrifugation and rinsing with distilled water 5-6 times.
DNA was extracted using the QIAamp® DNA stool mini kit (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.

3.3.1.1. Oocyst rupture

To increase the success of DNA extraction from oocysts of *Cryptosporidium* in stool samples, they were treated with the ASL lysis buffer provided in the QIAGEN kit and subjected to a freezing and thawing process according to the following procedure.
A pea sized (180 to 200 mg) portion of each solid or semi-solid stool or 100 µl of watery stool samples were added to a 1.5 ml Eppendorf tube containing 500 µl of ASL buffer. The samples were mixed by vortexing for 30 seconds until they were completely emulsified and

homogenised. Afterwards, the samples were exposed to six cycles of thawing-freezing at +80°C in a heating block and –80°C in a freezer for 15 and 30 minutes, respectively (Kim *et al.*, 1992).

3.3.1.2. Use of QIAamp® DNA stool mini kit

800 µl of ASL buffer were added to the 500 µl stool freeze-thaw extract and mixed well. The suspensions were heated for 5 minutes at 70° C. The tube was centrifuged at 13000 rpm for 1 minute and 1.2 ml supernatant mixture containing protein and nucleic acids was transferred to a new 2 ml micro-centrifuge tube. Potential PCR inhibitors were then removed from the supernatant by adding 1 Inhibit EX tablet to the sample. The tube was left for 1 minute at room temperature and then centrifuged at 13000 rpm for 3 minutes. 200 µl of the supernatant was transferred to a new tube and mixed with Proteinase K and AL buffer to digest the proteins. The tube was incubated at 70°C for 10 minutes after 15 seconds vortexing. Then, 200µl ethanol (100%) was added to the sample, vortexed and then applied to QIA amp® column tubes which were centrifuged at 13000 rpm for 1 minute. Digested proteins and excess enzyme were then washed off by application of the AW1 and AW2 buffers, the DNA remained 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. Finally, DNA was stored at -20° C until it was used for PCR assays.

3.3.2. PCR of *Cryptosporidium* DNA

3.3.2.1. Nested PCR for the 18S rRNA gene

A nested PCR was used to amplify the *Cryptosporidium* 18S rRNA gene using the primer sets AL 1687/1691 and AL 3032/1598 (detailed in Table 3.2) which amplify 1.3 and 0.83 kb segments, respectively; primer sequences and reaction conditions are adapted from Xiao *et al.* (1999b). The accession number in Gen Bank, for 18S rRNA gene of *C. hominis* (*C. hominis* strain HCNV4) is AF093489 and was used for comparative purposes. This method has been shown to provide highly specific information for species assignment and genotypic identification of *Cryptosporidium* (Xiao *et al.*, 1999a; Xiao *et al.*, 1999b).

Procedure and reagents

10X PCR buffer	Perkin Elmer [PE] Applied Biosystems, 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
Primary primers AL1687 and AL1691	Genosys Oligonucleotides, Sigma Genosys Ltd, Pamsford, Cambridgeshire, UK
Secondary primers AL3032 and AL1598	Genosys Oligonucleotides, Sigma Genosys Ltd, UK

Table 3.2. Primer sequences and positions on the complete 18S rRNA gene.

Primers	Nucleotide Position	Sequences
Primary		
AL1687	156-175	5'-TTC TAG AGC TAA TAC ATG CG-3'
AL1691	1455-1475	5'-CCC TAA TCC TTC GAA ACA GGA-3'
Secondary		
AL3032	193-218	5'-GGA AGG GTT GTA TTT ATT AGA TAA AG-3'
AL1598	1008-1029	5'AAG GAG TAAGGA ACA ACC TCC A-3'

The PCR reaction was carried out using the following components:

Primary PCR master mix

Reagents

10x Perkin Elmer buffer	5.0µl
2.5mM dNTPs	8.0µl (200 µM each)
25mM $MgCl_2$	12.0 µl (6mM final)
AmpliTaq DNA polymerase	0.50µl (2.5 U)
Primer AL 1687	2.50µl (100nM)
Primer AL 1691	2.50µl (100nM)
DNA template	2.0µl
1% BSA	1.0µl
HPLC water	Made up to final volume of 50µl

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

The amplification

The primary PCR master mixes were prepared in a clean laminar flow cabinet and dispensed into 200 µl PCR Eppendorf tubes using specific pipettes and filter-protected tips. All PCR runs included positive and negative controls. The positive control was obtained from The Department of Medical Microbiology and Genitourinary Medicine, The University of Liverpool. HPLC water was used as negative control. Disposable gloves were used and changed regularly for all procedures.

On a separate bench, DNA test templates were added to each tube. The samples were thoroughly mixed by vortexing, followed by centrifugation for about 30 seconds prior to loading into a Techne Thermal cycler (Techne Ltd., Cambridge, UK) using the following conditions.

The PCR cycling conditions

Initial denaturation	94 °C for 3 minutes

for 35 cycles	94 °C for 45 seconds
	55 °C for 45 seconds
	72 °C for 1 minute
Final extension at	72°C for 7 minutes

The secondary PCR was carried out using the following reactions:

Reagents

10x Perkin Elmer buffer	5.0µl
2.5mM dNTPs	8.0µl (200 µM each)
25mM $MgCl_2$	6.0 µl (3mM final)
AmpliTaq DNA polymerase	0.50µl (2.5 U)
Primer AL 1598	2.50µl (200nM)
Primer AL 3032	2.50µl (200nM)
Primary PCR product	2.0µl
1% BSA	1.0µl
HPLC water	Made up to final volume of 50µl

The main difference between the primary and secondary PCR was the use of 6mM $MgCl_2$ in the primary reaction, compared to 3mM $MgCl_2$ in the secondary PCR. This is to yield more primary PCR products, where the magnesium ions interact with the sugar-phosphate backbone of nucleic acids and influence the activity of Taq DNA polymerase (McPherson & S.G., 2000). The cycling conditions were exactly same as for the primary PCR.

3.3.2.2. Agarose gel electrophoresis

Reagents and equipment	
Agarose Ultra Pure	Life Technologies, Paisley, Scotland
Ethidium bromide	Sigma Chemicals, UK
1kb DNA ladder	Life Technologies, UK
Electrophoresis tank	Horizon 11014 Gibco BRL, Life Technologies, Scotland

Gel Documentation software	BioRad, Hertfordshire, UK
Tris-borate(TBE) buffer	5x TBE buffer: 54g Tris base, 27.5g Orthoboric acid, 20ml 0.5 mM EDTA pH=8.0 distilled water to 1 litre

Procedure

The presence and size of the PCR products were determined by agarose gel electrophoresis. A 2% agarose gel was prepared by mixing 2g of agarose powder and 100 ml of 0.5x TBE buffer. The mixture was heated in a microwave oven for 2 minutes or the minimum time required for all the agarose to dissolve. The solution was cooled for 3-5 minutes to 50-60 °C. Then, 2µl of ethidium bromide (0.05% w/v) was added to the mixture. It was then mixed thoroughly and poured into the Plexiglas gel support system with the comb in position. The gel was left at room temperature for about 30 minutes to solidify completely. The comb was removed and the gel was placed in the electrophoresis chamber and a sufficient volume of TBE 0.5x buffer was added to the chamber to just cover the surface of the gel.

2µl of loading buffer (containing bromophenol blue 0.05%) was mixed with 10µl of the secondary PCR products and this was loaded into the wells. Positive and negative controls were also included in each run. After loading the samples, the gel apparatus was connected to an electrical power supply and 150 volts applied for 45 minutes. After completing the electrophoresis, the gel was removed and placed on a UV light-box and photographed. PCR products were sized according to the migration of the standard ladder. Positive samples yielded approximately 840 base pair (bp) PCR fragments. The products were subsequently sequenced.

3.3.3. Restriction Fragment Length Polymorphism (RFLP) for the 18S rRNA

Restriction Fragment Length Polymorphism (RFLP) analysis of the secondary PCR product was done by digesting with *Ssp*1 for species identification and with *Vsp*1 for genotyping of *Cryptosporidium* species as described previously(Xiao *et al.*, 1999b).

Enzymes

All restriction enzymes which were used in RFLP procedure are shown in Table 3.3.

Table3.3. Restriction endonucleases and cleavage positions.

Enzyme	Biological source	Restriction site
*Ssp*1	*Sphaerotilus* spp	AAT ↓ ATT
		TTA ↑ TAA
*Vsp*1	*Arthrobacter* spp	AT ↓ TAAT
		TAAT↑ TA

Manufacture source: Boehringer, Mannheim, Germany.

The restriction endonuclease reaction

***Ssp*1**

*Ssp*1 20 U	1.0µl
10x Enzyme Buffer	2.5µl
HPLC water	11.5µl
2nd PCR product	15.0µl
Total volume	30.0µl

***Vsp*1**

*Vsp*1 20 U	1.0µl
10x Enzyme Buffer	2.5µl
HPLC water	11.5µl
2nd PCR product	15.0µl
Total volume	30.0µl

RFLP analysis of the secondary PCR product was done by digesting 15 µl of PCR product in a 30 µl total reaction volume at 37°C for 60 minutes. Digestion products were separated on 2% agarose gel and visualized by ethidium bromide staining as described in section 3.3.2.2. Expected size of products (in base pair) is shown in table 3.4.

Table 3.4. Expected size of products (in base pair) of the RFLP of the 18S rRNA gene of *Cryptosporidium* spp .

Species	Source	PCR product size (bp)	*Ssp*1 digestion (bp)	*Vsp*1 digestion (bp)
C. hominis	Humans	837	111,254,449	70,104,561
C. parvum	Cattle	834	108,254,449	104,628
C. felis	Cat	864	15,33,390,426	104,182,476

Adopted from Xiao *et al.*, (1999b).

3.3.4. Nested PCR for GP60 gene

For the sub-typing of *C. hominis* and *C. parvum*, the GP60 gene was amplified by nested PCR as previously described by Zhou *et al.*, (2003). Accession number AY262031 in GenBank provided the GP60 gene of *C. hominis* (isolate 2623, allele Ib) for comparison.

The majority of samples yielded a PCR product of about 350-480bp. The products were subsequently sequenced. Information about the primers that were used in this study is shown in Table 3.5.

Table 3.5. Primers sequences and positions on the complete GP60 gene.

Primers	Nucleotide Position	Sequences
Primary		
LX001 F1	4-21	5'-ATA GTC TCG CTG TAT TC-3'
LX002 R1	906-922	5'GCA GAG GAA CCA GCA TC-3'
Secondary		
LX003 F2	9-27	5'-TCC GCT GTA TTC TCA GCC-3'
LX004 R2	480 497	5'-GAG ATA TAT CTT GGT GCG-3'

Primers were synthesized by Genosys Oligonucleotides, (Sigma Genosys Ltd., UK).

The PCR reaction was carried out using the following components:

Primary PCR master mix

Reagents

10x Perkin Elmer buffer	5.0µl
2.5mM dNTPs	8.0µl
25mM $MgCl_2$	6.0µl
AmpliTaq DNA polymerase	0.50µl (2.5 U)
LX001 F1	1.25µl (100nM)
LX002 R1	1.25µl (100nM)
DNA template	1.0µl

1% BSA	2.0µl
HPLC water	Made up to final volume of 50µl

The amplification was carried out as described in section 3.3.2.1. using the following cycling conditions.

The PCR cycling conditions

Initial denaturation	94 °C for 3 minutes
for 35 cycles	94 °C for 45 seconds
	50 °C for 45 seconds
	72 °C for 1 minute
Final extension at	72 °C for 7 minutes

The secondary PCR was carried out using the following reaction:

Reagents

10x Perkin Elmer buffer	5.0µl
2.5mM dNTPs	8.0µl
25mM $MgCl_2$	6.0µl
AmpliTaq DNA polymerase	0.50µl (2.5 U)
LX003 F2	2.50µl (100nM)
LX004 R2	2.50µl (100nM)
Primary PCR product	2.0µl
1% BSA	1.0µl
HPLC water	Made up to final volume of 50µl

Cycling conditions were exactly same as for the primary PCR. 10µl of the secondary PCR products were separated on 2% agarose gel and visualized by ultraviolet trans-illumination having stained the gel with ethidium bromide. The rest of the secondary products were stored at -20 °C for future sequencing.

3.3.5. Nested PCR for HSP70 gene

A fragment, about 1200 bp in size, in the last half of the HSP70 gene of *Cryptosporidium* species was amplified followed by DNA sequencing for a sub-genotype study.

The primers were designed by Gatei and others (Gatei *et al.*, 2006) and are described in Table 3.6.

Table 3.6. Primer sequences and positions on the complete HSP70 gene.

Primers	Nucleotide Position	Sequences
Primary		
HSP70 F1	922-941	5′-ACT CTA TGA AGG TAT TGA TT-3′
HSP70 R1	2074-2051	5′-TTA GTC GAC CTC TTC AAC AGT TGG-3′
Secondary		
HSP70 F2	945-962	5′-CAG TTG CCA TCA GTA GAG-3′
HSP70 R2	2060-2039	5′-CAA CAG TTG GAC CAT TAG ATC C-3′
Internal		
HSP70 INT	1831-1849	5′-GGA CGA GTT TGA ACA TCA A-3′

Primers were synthesized by Genosys Oligonucleotides, (Sigma Genosys Ltd., UK).

A nested PCR strategy was used to amplify the HSP70 gene using the following reactions:

Primary PCR master mix

Reagents

10x Perkin Elmer buffer	5.0µl
2.5mM dNTPs	8.0µl
25mM $MgCl_2$	12.0µl
AmpliTaq DNA polymerase	0.50µl (2.5 U)
HSP70 F1	1.25µl (100nM)
HSP70 R1	1.25µl (100nM)
DNA template	1.0µl
1% BSA	2.0µl
HPLC water	Made up to final volume of 50µl

The amplification was carried out as described in section 3.3.2.1. using the following cycling conditions.

The PCR cycling conditions

Initial denaturation	94 °C for 3 minutes
for 35 cycles	94 °C for 45 seconds
	55 °C for 45 seconds
	72 °C for 1 minute
Final extension at	72 °C for 7 minutes

The secondary PCR was carried out using the following reaction mixture:

Reagents

10x Perkin Elmer buffer	5.0µl
2.5mM dNTPs	8.0µl
25mM $MgCl_2$	6.0µl
AmpliTaq DNA polymerase	0.50µl (2.5 U)
HSP70 F2	2.50µl (100nM)
HSP70 R2	2.50µl (100nM)
Primary PCR product	2.0µl
1% BSA	1.0µl
HPLC water	Made up to final volume of 50µl

The cycling conditions were exactly same as in the primary PCR. 10µl of the secondary PCR products were separated on a 2% agarose gel and visualized by ultraviolet trans-illumination having stained gel with ethidium bromide. The rest of the secondary products were stored at -20°C for future sequencing.

3.4. Sequencing study

All secondary PCR products of the three genes: 18S rRNA, GP60 and HSP70 were sequenced directly by using reverse and forward primers (and internal primer in case of the HSP70 gene) using an automated DNA sequencer (ABI 3100 Automatic Sequencer, Warrington UK).

3.4.1. Statistical and sequence analysis

The data in chapter 4 were analysed using the Microsoft Excel and Epi Info version 6 programmes.

Kappa value was calculated using a program on the website:
http://www.dmi.columbia.edu/homepages/chuangj/kappa/

The ChromasPro programme version 1.3 (2003, Technelysium Pty.Ltd., Australia) was used to read all the amplicon sequences. Manual editing of the consensus sequences was

performed using DNASTAR software version 4.05 (1993-2000), Edit2 and SeqManII. To confirm the identity of the sequences from the GenBank, Blast Local Alignment Search Tool (BLAST) searches National Centre for Biotechnology (NCBI) were undertaken (Table 3.7). Multiple alignments of the DNA sequences were done with the CLUSTALX program (EMBL, Heidelberg, Germany).

The DNA distance based Neighbor Joining (NJ) analysis was performed on the aligned sequences by using the phylogenetic analysis software PHYLIP version (3.6). PHYLIP is a comprehensive phylogenetic analysis package created by Joseph Felsenstein at the University of Washington.

The drawing of dendrograms was performed with TREECON for windows version 1.3b, a software package developed by Yves Van de Peer for the construction and drawing of phylogenetic trees based on distance data.

Table 3.7. **The sequence used for comparison from the GenBank. (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi)**

Accession No	Locus	Species	Strain or isolate
EF611871	18S rRNA	*C. parvum*	Izatnagar
DQ656355	18S rRNA	*C. parvum*	IR-C2
EF570921	18S rRNA	*C. parvum*	CHZF1
Ay204238	18S rRNA	*C. parvum*	HMa
AF108864	18S rRNA	*C. parvum*	C1
EF570922	18S rRNA	*C. hominis*	CHZF2
AJ849464	18S rRNA	*C. hominis*	S119
AF481962	18S rRNA	*C. hominis*	UG502
AF093498	18S rRNA	*C. muris*	IDRH-13
AY738186	GP60	*C. parvum*	5635
EF025582	GP60	*C. parvum*	SH75
EF073049	GP60	*C. parvum*	HU-27
AY738194	GP60	*C. parvum*	8897
AY738192	GP60	*C. hominis*	7505
DQ665688	GP60	*C. hominis*	4332
DQ665689	GP60	*C. hominis*	4302
AY262031	GP60	*C. hominis*	2623
XM_625373	HSP70	*C. parvum*	Iowa II
AF221528	HSP70	*C. parvum*	11
DQ886260	HSP70	*C. hominis*	7070
EF591788	HSP70	*C. hominis*	CHZF2
DQ665696	HSP70	*C. hominis*	4332
AY380458	HSP70	*C. hominis*	A5
AF329189	HSP70	*C. meleagridis*	672

3.4.2. Cloning

The secondary PCR products of 18S rRNA gene of 8 mixed samples (Ardah: 3,12,64,73,101,130, Samtah 92 and Abu Areesh 28) were cloned into pGEM-T to investigate whether these isolates include novel species or mixed infection with different *Cryptosporidium* species.

Principle

The procedure involves ligation of the DNA fragment into a plasmid vector expressing selectable markers such as colour change and an antibiotic resistant ß-lactamase gene. The screening for the presence or absence of the functional ß-galactoside gene involves the addition of a lactose analogue named X-gal plus IPTG as an inducer of the enzyme. The former is broken down by ß-galactoside to a coloured deep blue product. Non-recombinant colonies, the cells of which synthesize ß-galactosidase, will be blue coloured. Recombinants will have a disrupted gene and be unable to make ß-galactosidase and thus produce white colonies. Only bacteria containing the vector with the PCR product inserted will grow as white colonies. Transformed bacteria have antibiotic resistance and white colonies while non-transformed colonies are blue. Transformed colonies are selected and grown in bacterial broth to yield enough copies of the test DNA. The DNA is then extracted from the transformed bacteria and purified for sequencing.

Procedure

The commercial pGEM-T cloning kit (Promega, Madison, USA) was used. The Test DNA was cloned into the pGEM-T plasmid vector and transformed into competent *Escherichia coli* TG2 cells.

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

RF1 Buffer

100mM $RbCl_2$	6.05 g	Sigma-Aldrich, UK
30mM K acetate	1.47 g	BDH, England
10mM $CaCl_2$	0.735 g	BDH, England
50mM $MnCl_2$	4.95 g	Sigma-Aldrich, UK
75 ml glycerol (15%)		BDH, England
		pH 5.8 with acetic acid

Made up to 500 ml with distilled water

RF2 Buffer

10M MOPS	1.05 g	Sigma-Aldrich, UK
75mM $CaCl_2$	5.5 g	
10mM $RbCl_2$	0.6 g	
75 ml glycerol (15%)		
		pH 6.5 with KOH

Made up to 500 ml with distilled water

Procedure

E.coli TG2 cells were made competent by incubating with buffer RF1 on ice for 15 minutes followed by centrifugation at 13000 rpm for 5 minutes. The resultant pellet was incubated on ice with buffer RF2 for 30 minutes. The competent bacteria were then dispensed into aliquots (200µl each) and stored at -20 °C until used.

3.4.2.2. Cloning of PCR products into pGEM-T

About 3µl of secondary PCR product was incubated with 2x ligation buffer, pGEM-T vector (1µl) and T4 DNA ligase (1µl) in a total volume of 10µl for 1 hour on the bench.

3.4.2.3. Transformation

The pGEM-T vector with the insert DNA was transformed into 200µl competent TG2 cells by incubation on ice for 1 hours, heating shocking at 42° C for 45 seconds, incubation on ice for 5 minutes and growth at 37° C in Luria broth (LB-10g Becto-tryptone, 5g Becto-yeast extract, 5 gNaC1 and 1 litre distilled water) for 45 minutes then the bacteria were plated onto LB-agar plates with the following reagents and grown at 37° C overnight.

Ampicillin	100µg/ml	Sigma-Aldrich, UK
X-gal (substrate)	80µg/ml	Calbiochem
(5-bromo-4-chloro-3 indolyl-ß-D galactopyranoside)		
IPTG (inducer)	200µg/ml	Sigma-Aldrich, UK

(Isopropyl-ß-D thiogalactopyranoside)

Colonies containing the vector with the target cloned PCR products were grown in 500 ml of Luria broth, overnight at 37°C in a 200 rpm shaker. A total of 64 transformed colonies for 18S rRNA (8 for each sample) were then screened by PCR with the primers previously mentioned in Table 3.2.

RFLP for the 18S rRNA gene was used to differentiate among infecting species in each sample. 16 representative transformed colonies, two of each sample that showed different restriction pattern with *Vsp*1 were chosen for recovery of the DNA from transformed bacteria for sequencing.

3.4.2.4. Recovery of the DNA from transformed bacteria

DNA was recovered from the transformed bacteria by using the QIAprep Spin Miniprep Kit Protocol (QIAGEN, UK). The test DNA was then recovered using a spin column membrane and a high salt buffer (all provided), that bind the DNA to the membrane. The DNA is then eluted from the membrane by using 50µl HPLC water. Finally, about 1 µg of the test DNA was aliquoted into an Eppendorf, labelled and sequenced with the following vector specific primers using automated DNA sequencers (ABI3100 Automatic Sequencer).
Vector specific primers:
M13-20 GTA AAA CGA CGC CCA GTG AG
M13-rev GGA AAC AGC TAT GAC CAT G

4. DETECTION OF *CRYPTOSPORIDIUM* SPP.

4.1. Introduction

Bright-field microscopy is one of the routine diagnostic procedures for diagnosis of cryptosporidiosis. Various acid-fast stains using hot or cold procedures have been developed including Ziehl-Neelsen and cold Kinyoun (Baxby *et al.*, 1984; Hart, 1999). With these methods, the stained oocysts stand out against a counterstained background. The safranin-methylene blue (SMB) technique (Baxby *et al.*, 1984) is based on a spore stain and pink oocysts stand out against the blue background (Hart, 1999). In a comparative study SMB proved to be more efficient than Ziehl-Neelsen in detecting oocysts (Baxby *et al.*, 1984). With any of these methods care must be taken not to confuse moulds, fungal spores or droplets of fat with oocysts. These methods may be useful for screening samples, but identification should be confirmed with more specific assays such as enzyme immunoassays (EIA/ELISA) (Schuster & Chiodini, 2001).

Several immunofluorescent assays and ELISA kits have become commercially available and are increasingly 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). However it is not clear whether the assays can detect all *Cryptosporidium* spp., and there is some variation in specificity and sensitivity of the different commercial kits (Bialek *et al.*, 2002).

PCR assays have been introduced as very sensitive methods to detect *Cryptosporidium* spp. In this study a nested PCR assay was established, targeting part of the 18S rRNA gene of this parasite. The present study used ZN and SMB microscopy and ELISA to identify positive stool samples from different sources for genotyping. This chapter describes the results of the screening methods and PCR of the 18S rRNA gene to identify *Cryptosporidium* spp. oocysts in stool specimens from Gizan and Maddina, Saudi Arabia.

4.2. Results

4.2.1. Microscopy

A total of 516 human stool samples (454 from patients and 62 from the comparison group) were screened by microscopy using both the ZN and SMB staining techniques as described previously in section 3.2., and the 279 animal samples were screened by the ZN staining technique only.

In the ZN staining method, *Cryptosporidium* oocysts appeared as red oval/round bodies, about 4-5 μm in diameter. They stained irregularly, often with darker red staining around the periphery (Figure 4.1). In the SMB method, oocysts appeared as vivid orange-pink bodies approximately 5 μm diameter, usually spherical or slightly ovoid, (Figure 4.2). At a magnification of 10,000× under the electron microscope, it was possible to observe and identify the internal and external fine structures of sporozoites and oocyst walls that are difficult to detect using an optical microscope (Figure 4.3).

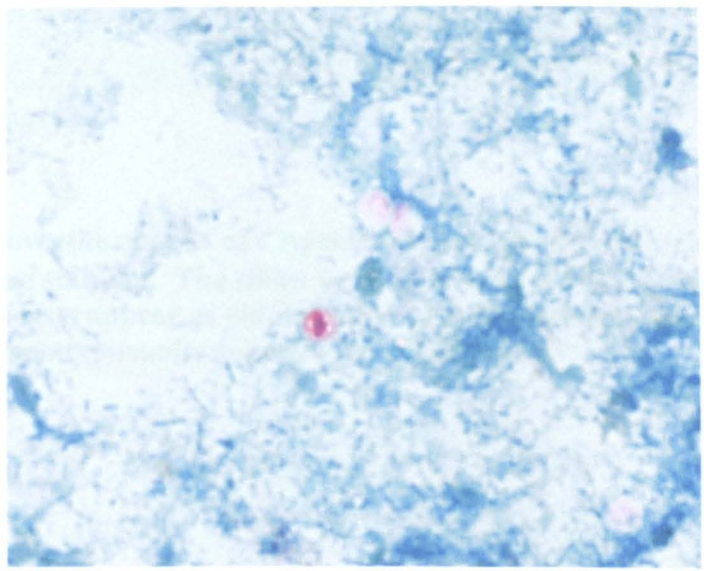


Figure 4.1. Shows the oocysts of *Cryptosporidium* of the Ziehl-Neelsen staining. The slides were examined at 1000 × magnification. Oocysts appear as red oval/round bodies, about 4-5 μm, while stain irregularity, often with darker red staining around the periphery

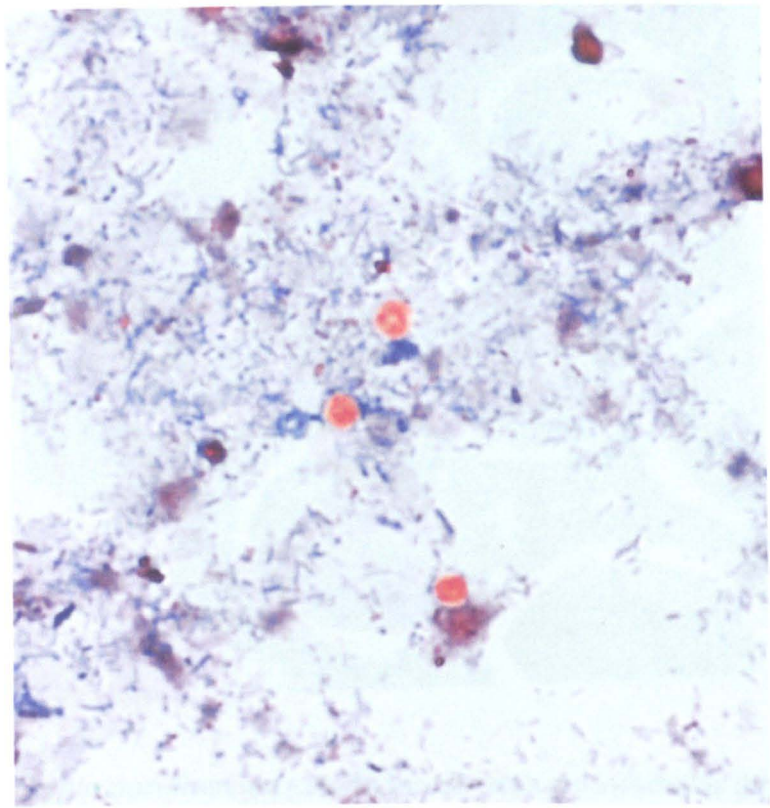


Figure 4.2 **Shows the oocysts of *Cryptosporidium* spp of the Safranin Methylene Blue staining. The slides were examined at 1000 × magnification. Oocysts appear as vidid orange-pink bodies approximately 5 μm diameter, usually spherical or slightly ovoid**

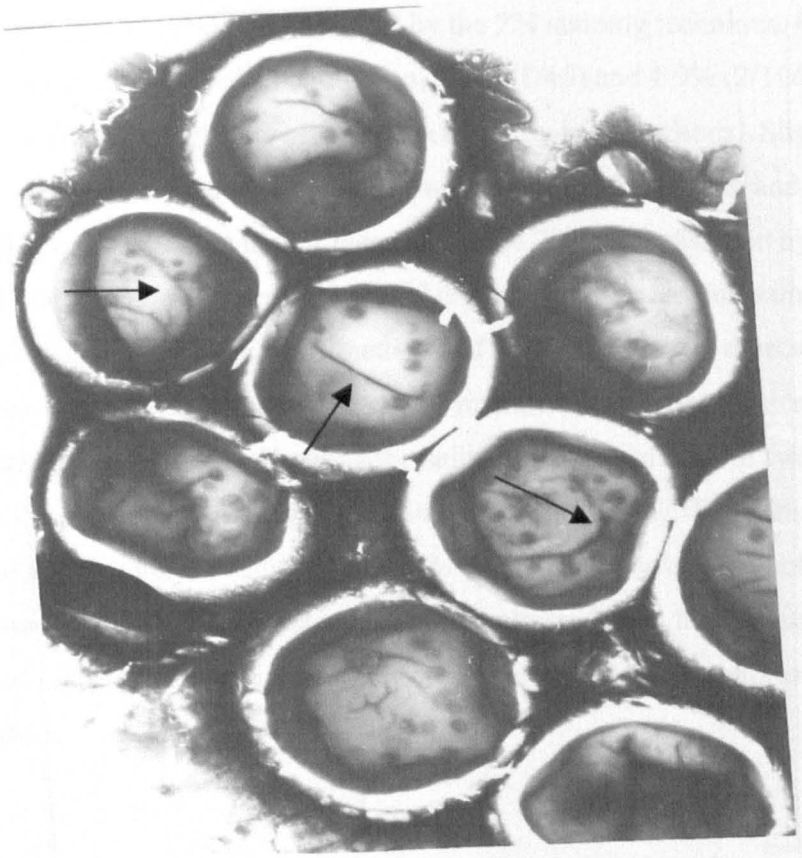


Figure 4.3. ***Cryptosporidium*** **oocysts under electron transmission thin section microscopy at 10,000 × magnification. The sutures through which the sporozoites leave the oocysts are visible (arrow) (c/o Brian)**

Faecal samples were collected from 4 hospitals, one primary health care centre, two schools and 5 animal farms in the Gizan area and from two governmental hospitals in the Maddina area of Saudi Arabia as described in section 3.1. *Cryptosporidium* oocysts were identified in 21(4.6%) of 454 human samples by ZN and 26 (5.7%) of 454 by SMB, while oocysts were identified in one sheep sample from Abu Areesh out of 62 (1.6%) samples using the ZN staining technique only (Table 4.1).

More positive samples were detected of the SMB staining method than of the ZN staining. Among human samples from Al-Ardah, there were different rates of positivity among the samples depending on the method used, 15/133 (11.3%) were detected by the

SMB method and 12/133 (9%) were detected by the ZN staining technique. Of the patient samples collected from Al- Khobah and Gizan, 2.2% (1/45) and 1.9% (2/106) were recorded as positive for *Cryptosporidium* respectively by both methods. Slightly different rates of positivity were found among samples from the Samtah and Abu Areesh areas, depending on the method used. Overall 5/106 (4.7%) were detected by the SMB method and 4/106 (3.8%) were detected by ZN technique in the Samtah samples, while 3/64 (4.7%) were detected by the SMB method and 2/64 (3.1%) were detected by ZN technique in the Abu Areesh samples. The SMB method was not used to screen the animal samples due to the need of heating the safranin for a total time of two minutes during which the stain boiled once which is time consuming especially with the large number of the study samples (Table 4.1). *Cryptosporidium* oocysts were not detected in any of the comparison group samples, which were collected from the two schools, the Samtah animal samples or Abu Areesh (cows). All the ZN positive samples were also positive by SMB.

4.2.2. Antigen-EIA/ELISA technique

All 1920 samples were examined by an Enzyme Immunoassay (EIA) which detects *Cryptosporidium* Specific Antigen (CSA) as described previously in section 3.2.3. This technique also checks the accuracy of the microscopy methods. The ELISA method (Figure 4.4) was positive in 45/454 (9.9%) samples from symptomatic human cases in Gizan (Table 4.1), while no positive results were obtained from the 62 comparison group samples. In Maddina, 58/1125 (5.2%) samples were positive by ELISA. Out of 795 samples from Gizan (human and animal samples) that were examined by both ELISA and light microscopy, 46 samples were identified as positive by ELISA compared to 27 positive samples seen to be positive by at least one of the microscopy staining methods. This indicates that the ELISA method is more sensitive than microscopy. The highest percentage of CSA identification was recorded from Al-Ardah , 26 out of 133 samples (19.5%), followed by 5/64 (7.8%) from Abu Areesh. 8/106 (7.5%) ELISA positive samples were identified from Samtah, 58/1125 (5.2%) from Maddina, 4/106 (3.8%) from

Gizan city and 2/45 (4.4%) from Al-Khobah. Among the animal samples, only one sheep sample was recorded as positive by ELISA, 1/279 (0.36%).

A few samples were detected as very weak positive by ELISA (O.D. ranged between 0.06 to 0.08) as just above the cut-off of absorbance (≥0.050). These samples were re-tested using the same technique and gave lower readings than the first time. They were also negative by 18S rRNA PCR and by electron microscopy and for these reasons they were designated as negative. These weak positive results by ELISA are probably false positive results of this test (Parisi & Tierno, 1995; Doing *et al.*, 1999). However, all the ELISA positive samples have been confirmed by either all 18S rRNA, GP60 and HSP70 *Cryptosporidium* genes or at least by one gene only.

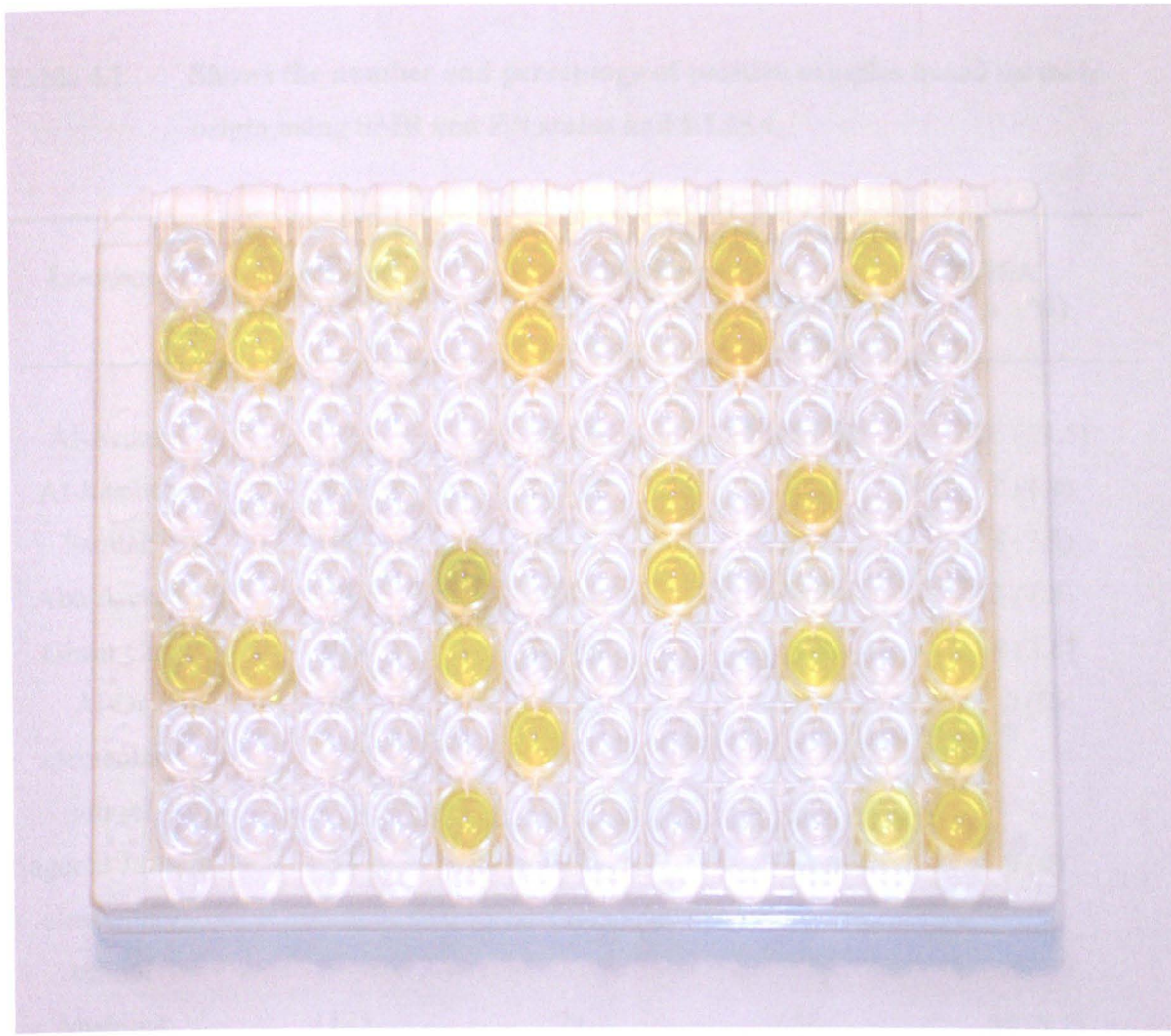


Figure 4.4. **An ELISA test plate with 96 wells. The yellow circles indicate positive reactions for positive samples, while the white circles indicate negative reactions for negative samples.**

Table 4.1. **Shows the number and percentage of positive samples based on their origin using SMB and ZN stains and ELISA.**

Location	No tested	No (%) Positive Microscopy		ELISA
		ZN n (%)	SMB (%) n (%)	n (%)
Al-Ardah	133	12 (9)	15 (11.3)	26 (19.5)
Al-Khobah	45	1 (2.2)	1 (2.2)	2 (4.4)
Samtah	106	4 (3.8)	5 (4.7)	8 (7.5)
Abu Areesh	64	2 (3.1)	3 (4.7)	5 (7.8)
Gizan City	106	2 (1.9)	2 (1.9)	4 (3.8)
Al-Or elementary school	29	0 (0)	0 (0)	0 (0)
Sager al Jazeera elementary school	33	0 (0)	0 (0)	0 (0)
Maddina	1125	N	N	58 (5.2)
Samtah (Cows)	64	0 (0)	N	0 (0)
Samtah (Sheep)	64	0 (0)	N	0 (0)
Samtah (Goats)	57	0 (0)	N	0 (0)
Abu Areesh (Sheep)	62	1 (1.6)	N	1 (1.6)
Abu Areesh (Cows)	32	0 (0)	N	0 (0)
Total	**1920**	**22**	**26**	**104**

N= the technique was not used

4.3. Comparison of detection methods for *Cryptosporidium* spp

Among 454 stool specimens of patients from Gizan which were analysed by microscopy oocysts were identified in 21 (4.6%) samples by ZN and 26 (5.7%) by SMB, the strength of agreement between these two methods was almost perfect (Kappa value=0.88) (Table 4.2). McNemar's test shows that the difference between the two methods was not quite statistically significant.

Table 4.2 Comparison of microscopy screening for *Cryptosporidium* oocysts using the SMB and ZN staining methods.

Method	SMB		Total
ZN	Positive N (%)	Negative N (%)	
Positive N (%)	21 (4.6%)	0 (0%)	21
Negative N (%)	5 (1.1%)	428 (94.3%)	433
Total	26	428	454

Kappa value=0.88 (strength of agreement between the two methods is almost perfect). McNemar Test: Chi^2=3.2, P=0.0736

When the ELISA technique was performed on the same samples, the detection rate of *Cryptosporidium* oocysts was 45 (9.9%) which is higher than the 4.6% and 5.7% by the two different staining methods.

The strength of agreement between these two methods was substantial (Kappa value=0.71) Table 4.3). McNemar's test shows that there is a significant higher number diagnosed by ELISA (P<0.0001).

Table 4.3 **Comparison of microscopy and ELISA in screening for *Cryptosporidium* oocysts.**

Method	**ELISA**		**Total**
SMB stain	Positive N (%)	Negative N (%)	
Positive N (%)	26 (5.7%)	0 (0%)	26
Negative N (%)	19(4.2%)	409 (90%)	428
Total	45	409	454

Kappa value=0.71 (strength of agreement between the two methods is substantial). McNemar Test: Chi^2=17.053, P<0.0001

21 samples were positive by all three screening methods which were used in the Gizan study (ELISA, SMB and ZN) while 5 samples were positive by both the SMB staining technique and ELISA. A total of 19 positive samples were identified by ELISA only. No sample was positive by microscopy but negative by ELISA which means that the agreement in specificity between the two methods was 100% but ELISA was more sensitive in detecting *Cryptosporidium* oocysts (p=0.026).

PCR of the 18SrRNA *Cryptosporidium* gene was performed on all samples from Gizan that were positive by microscopy and/or ELISA. Few negative random samples were included and they were negative also by this method. All the 27 samples that were positive by microscopy were also positive by 18S rRNA PCR, except one sample from Al-Ardah (sample no 115), which was confirmed subsequently by GP60 PCR only.

Of 104 positive samples of *Cryptosporidium* spp. which were identified by ELISA test, 101 samples yielded a PCR product and only 3 samples from Al-Ardah (Ar 37, Ar 84 and Ar 115) failed to produce amplicons for the 18S rRNA gene in spite of repeating the 18S rRNA PCR many times. Two of these samples (Ar 37 and Ar 84) were positive only by ELISA and one sample (Ar 115) was positive by both ZN and SMB staining methods and ELISA. Subsequently, all the three negative samples with 18S rRNA gene yielded a PCR product for the GP60 gene and one sample (Ar 84) yielded a PCR product for the HSP70 gene

The gold standard detection method for *Cryptosporidium* spp. in this study was ELISA because it was able to detect all the positive samples that have been detected by microscopy and screening PCR (Table 4.4).

Table 4.4 Comparison between microscopy, ELISA and PCR of 18S rRNA gene for detection of *Cryptosporidium*.

N=46	
Detection method	**No.of positives**
Microscopy	27
ELISA	46
18S rRNA PCR	43

4.4 Patient characterization in Gizan

A total of 454 stool specimens were collected from children suffering from gastroenteritis from different parts of the Gizan Area between October 2004 and February 2005 as described previously in sections 3.1 & 3.1.1.1. A total of 45 (9.9%) of these specimens were positive for *Cryptosporidium* by enzyme immunoassay.

The median age of the positive cases was12 months. As expected, *Cryptosporidium* infection was more common (71.1%) in children less than two years of age ($p<0.01$), and about half (48.9%) of the detected positives were among children in the first year of life ($p<0.01$) (Table 4.5). The highest number of cases were observed in patients (0-5) months of age (48%) and, nearly two thirds of *Cryptosporidium* infections were detected among children between 0-18 months. The prevalence of cryptosporidiosis was only 28.9% in children older than two years. The age range of positives ranged from 15 days to 120 months (figure 4.5). There was a significant relationship between young age and cryptosporidiosis.

Table 4.5 Prevalence of cryptosporidiosis by age, as detected by ELISA, in the Gizan area.

Age group (months)	Number of samples	Positives	Prevalence	Cumulative frequency
0-5	25	12	48%	12
6 to 11	27	10	37%	22
12 to 17	40	8	20%	30
18-23	33	2	6%	32
24-35	60	5	8%	37
36-47	64	3	5%	40
48-59	29	1	3%	41
60-72	53	1	2%	42
72 and Up	123	3	2%	45
Total	**454**	**45**	**10%**	**45**

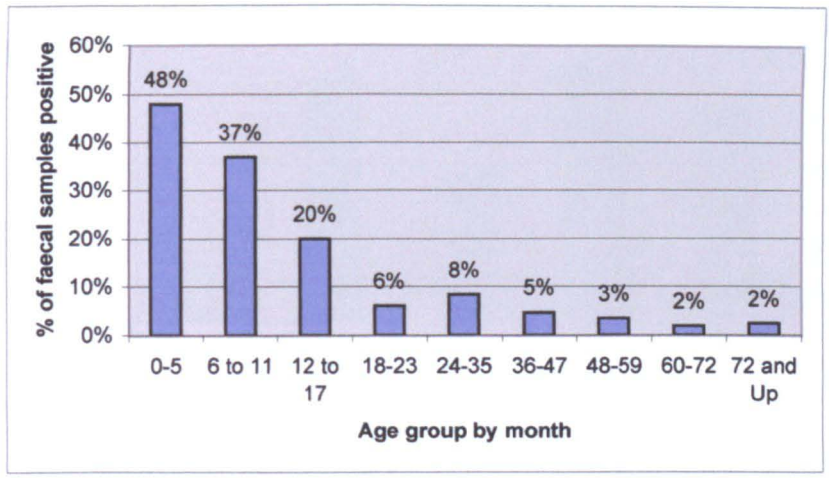


Figure 4.5. Prevalence of cryptosporidiosis by age in Gizan area

Cryptosporidium infection was present throughout the 5 months of the study period (October – February), with maximum prevalences during the cooler months (December – February) (Table 4.6). The peak of infection was in January with 13% of the diarrhoeal cases being due to *Cryptosporidium*, followed by February (11%). Moreover, 89% of the cases of cryptosporidiosis detected in Gizan occurred from December to February, with lower numbers occurring during October and November (Figure 4.6). This trend was statistically significant ($p<0.01$).

Table 4.6. Prevalence of cryptosporidiosis by month in Gizan area

Month	Total Samples	Positive	Prevalence	Cumulative frequency
October	28	1	4%	1
November	33	4	12%	5
December	160	12	8%	17
January	116	15	13%	32
February	117	13	11%	45
Total	**454**	**45**	**10%**	**45**

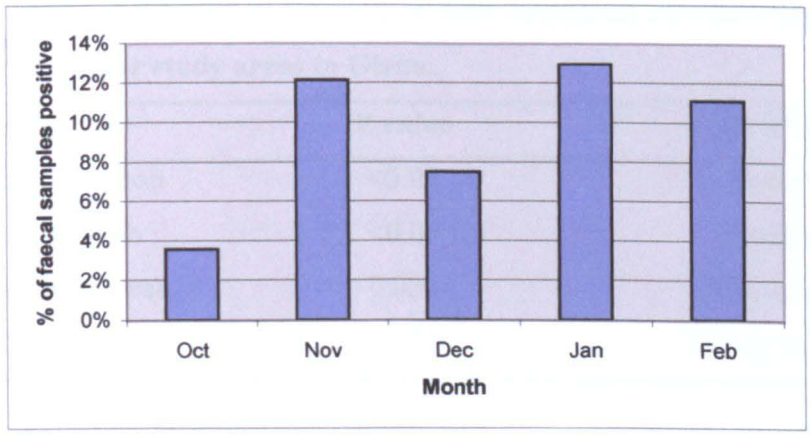


Figure 4.6. Prevalence of cryptosporidiosis by month in Gizan area

Fifty three percent (n=239) of the samples came from females, but identical proportion of males (21/215, 10%) and females (24/239, 10%) were positive for cryptosporidiosis.

The highest prevalence of cryptosporidiosis was in Al-Ardah (20%) followed by Samtah and Abu-Areesh (8%), while the lowest prevalences were in Al-Khobah and Gizan (4%) (Table 4.7) (Figure 4.7). The prevalence of cryptosporidiosis in Al-Ardah was significantly higher than in Al-Kobah, Samtah, Abu Areesh and Gizan and p values are shown in Table 4.8.

Table 4.7 Prevalence of cryptosporidiosis by area in Gizan area

Area	Median age (month)	Total samples	Positive	Prevalence
Al-Ardah	24	133	26	20%
Al-Khobah	34	45	2	4%
Samtah	38	106	8	8%
Abu Areesh	43.5	64	5	8%
Gizan City	38	106	4	4%
		454	45	10%

Table 4.8. Type of relation in *Cryptosporidium* incidence between Al-Ardah and the other study areas in Gizan.

Areas compared	P value	Type of relation
Al-Ardah to Al-Khobah	<0.05	Significant
Al-Ardah to Samtah	<0.05	Significant
Al-Ardah to Abu Areesh	0.056	Not significant
Al-Ardah to Gizan	<0.01	Highly significant

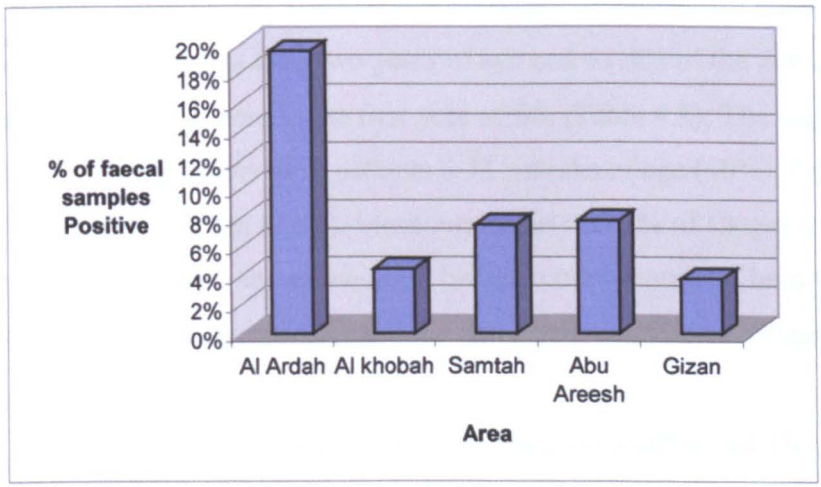


Figure 4.7. Prevalence of cryptosporidiosis by Area in Gizan

4.5 Patient characterization in Maddina

1125 stool specimens were collected from children under five years of age with acute, dehydrating diarrhoea who were either admitted to the hospitals as inpatients (620), or given oral rehydration therapy (ORT) and treated as outpatients (505). This was done between April 2004 and April 2005 as described previously in section 3.1 & 3.1.1.1.

58 (5.2%) of these specimens were positive for *Cryptosporidium* spp. by enzyme immunoassay. The age spectrum of cases ranged from 5-60 months with a median age of 12 months. As expected, *Cryptosporidium* spp. infection was more common (63.8%) among children less than two years of age and 41.4% of the detected positives among children were in the first year of life (Table 4.9). The highest numbers of cases were observed in patients 6-11 months of age (40%), followed by patients 24-35 months of age (22%). Moreover, nearly 55.2% of *Cryptosporidium* spp. infection was detected among children between 0-18 months. There was a significant relationship between younger age and cryptosporidiosis. (Figure 4.8).

Table 4.9 Prevalence of cryptosporidiosis by age, detected by ELISA, in Maddina.

Age Groups (month)	Number of samples	Positives	Prevalence	Cumulative frequency
0-5	130	1	1%	1
6-11	212	23	11%	24
12-17	123	8	7%	32
18-23	116	5	4%	37
24-35	218	13	6%	50
36-47	160	2	1%	52
48-59	91	3	3%	55
>60 Months	75	3	4%	58
Total	**1125**	**58**	**5%**	**58**

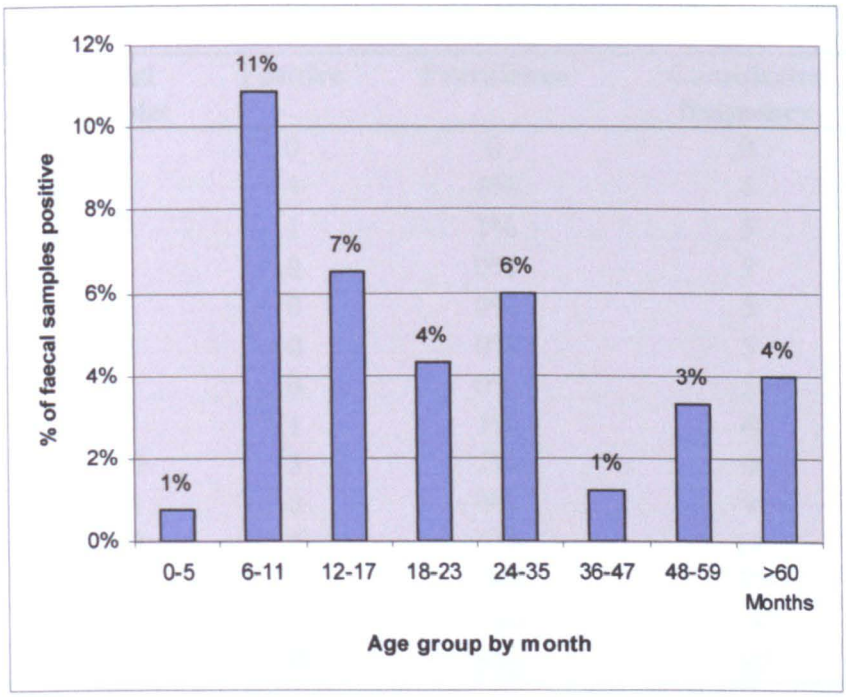


Figure 4.8 Prevalence of cryptosporidiosis by age in Maddina.

Cryptosporidium infection was only detected during 7 months of the study period (May, June, November, December, February, March and April) and was absent in the other months, with maximum prevalences during the cooler months, February and March (Table 4.10). The peak of infection was March when 38% of the diarrhoeal disease cases were due to *Cryptosporidium* spp, followed by February (5%). Overall, 82.7% of the *Cryptosporidium* spp detected in Maddina were detected during February and March. (Figure 4.9). The prevalences in the period between February and April (spring) were compared to the prevalences in the other months of the study, and were significantly higher ($p<0.01$).

Table 4.10 Prevalence of cryptosporidiosis by month in Maddina

Month	Total samples	Positive	Prevalence	Cumulative frequency
April	48	0	0	0
May	96	4	4%	4
June	75	1	1%	5
July	79	0	0%	5
August	56	0	0%	5
September	83	0	0%	5
October	88	0	0%	5
November	67	1	1%	6
December	129	3	2%	9
January	96	0	0%	9
February	103	5	5%	14
March	114	43	38%	57
April	91	1	1%	58
Total	**1125**	**58**	**5%**	**58**

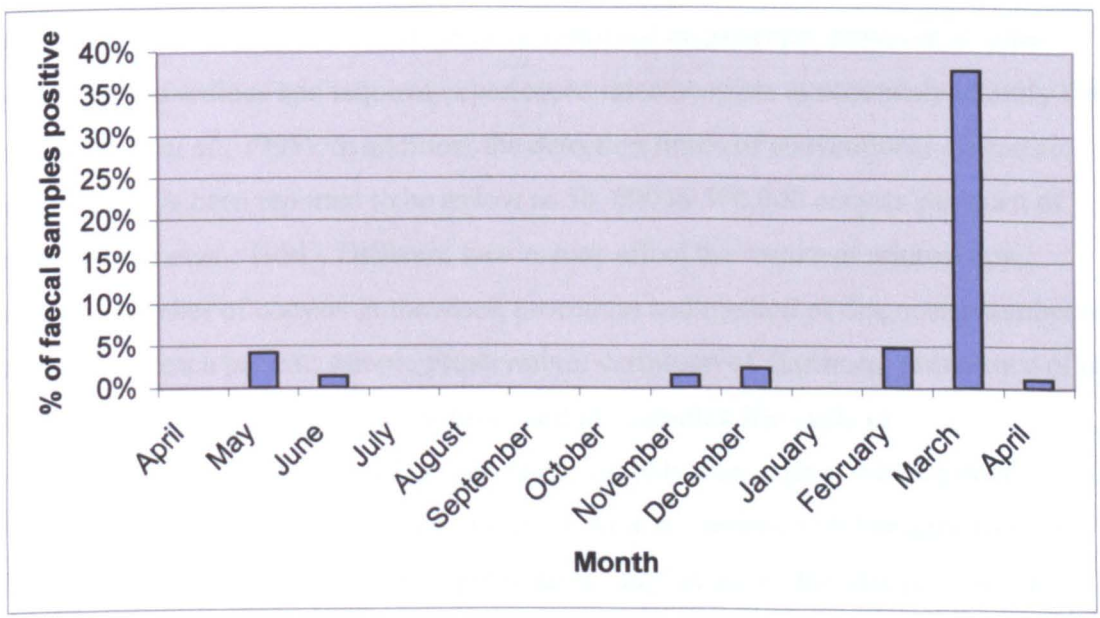


Figure 4.9 Prevalence of cryptosporidiosis by month in Maddina

The prevalence of *Cryptosporidium* in males was 35/608 (5.8%) compared to 23/517 (4.4%) in females, but the difference was not statistically significant (p=0.3). Diarrhoea due to *Cryptosporidium* spp. occurred in 22 out-patient cases compared to 36 in-patient cases with prevalence of 22/505 (4%) and 36/620 (6%) respectively (p=0.3).

4.6 Discussion

This chapter compares methods for screening stool samples for *Cryptosporidium* spp. from human and animals including sheep, goats and cows from Gizan and human samples only from Maddina, Saudi Arabia, the methods used were SMB, ZN, ELISA and 18S rRNA PCR.

Conventional methods for identification of *Cryptosporidium* oocysts include examination of faecal smears with acid-fast stains such as Ziehl-Neelsen, which are commonly used by diagnostic facilities. Conventional microscopy, however, is time-consuming and tedious and requires experienced microscopists to accurately identify the oocysts (Kehl *et al.*, 1995). In addition, the detection limits of conventional diagnostic techniques have been reported to be as low as 50, 000 to 500,000 oocysts per gram of faeces (Weber *et al.*, 1991). Different factors may affect the results of microscopy, including: number of oocysts in the stool; procedure and method of diagnosis; number of samples from each patient; sample preservative; definition of diarrhoea; prevalence of the parasite in the community; host condition; and the complex life cycle of *Cryptosporidium*, the oocysts being present during only one stage of development (Fayer *et al.*, 1997). Hence the parasite may infect a host in the absence of detectable oocysts. Therefore, traditional parasitological procedures, such as use of formalin ethyl-acetate concentrations with examination of iodine stained preparations and trichrome staining, are not adequate. Special staining techniques, such as ZN and SMB, or ELISA which enhance the ability to screen large numbers of samples for the presence of *Cryptosporidium* antigen, must be applied.

Immunologically based detection methods have been developed for use in both clinical and environmental monitoring. However, antigenic variability within clinical isolates of *Cryptosporidium* spp can result in some infections remaining undetected, and there are conflicting reports as to the sensitivity of immunodetection methods over microscopy (Morgan *et al.*, 1998)

Cryptosporidiosis is usually diagnosed by the recognition of oocysts in stained faecal smears prepared, either directly from faeces or after concentration procedures (Scott *et al.*, 1995; Guyot *et al.*, 2001). In this study, the direct smear preparation was selected because it is simple and efficient procedure for examination of faeces (Eberhard *et al.*, 1997), although using a concentration method before examination was recommended (Scott *et al.*, 1995).

Out of a total of 454 human samples from patients in Gizan, *Cryptosporidium* oocysts were identified in 21 (4.6%) and 26 (5.7%) by the ZN and SMB staining methods respectively (Table 4.1). This finding is in agreement with results obtained from Baxby *et al* (1984) where 26 cases of cryptosporidiosis were diagnosed by SMB staining method, but only 19 were detected by the acid-fast stain in 36 cases infected with *Cryptosporidium* spp. However the agreement between the two methods was almost perfect (Kappa value=0.88) (Table 4.2). A few studies have reported prevalences or the positivity rate of *Cryptosporidium* infection in other areas in Saudi Arabia using bright field microscopy of between 1% and 32% (Khan *et al.*, 1988; Al-Braiken *et al.*, 2003) (Table 2.1). Minor differences between results obtained by ZN and SMB were found especially in Al-Ardah where the majority of *Cryptosporidium* oocysts were detected, i.e. 12/133 (9%) and 15/133 (11.3%) *Cryptosporidium* positive cases were detected there using ZN and SMB respectively. This finding is in agreement with high positivity rate of cryptosporidiosis (3-11%) which was reported in children with gastro-enteritis and HIV/AIDS patients from Malawi (Pavone *et al.*, 1990; Cranendonk *et al.*, 2003). But in the other areas compared to Al-Ardah, no clear difference was seen between the two staining methods. This is the first study looking for *Cryptosporidium* spp. in Gizan, Saudi Arabia.

Among 279 samples from domestic animals including sheep, goats and cows were screened by ZN staining technique and ELISA , only one positive sample was detected from a sheep (1/279=0.36%). There are no published reports describing the prevalence of cryptosporidiosis among animals in Saudi Arabia except one report from the Al-Ahsa region that described the prevalence of the parasite in white cheeked bulbuls (Al-Atiya *et al.*, 2004). This described a prevalence of 28.6% and it is not in agreement with our study results.

These findings could have various explanations such as:

i) Using one sample for each case. It is well established that the number of oocysts can be quite variable, even in liquid stools, and that, because of the intermittent nature of oocysts excretion by this parasite (Tzipori *et al.*, 1983; Navin & Juranek, 1984), multiple stool samples should be tested before a negative diagnostic interpretation is reported. Single-stool examination identified only 50% of infected individuals involved in an outbreak of waterborne cryptosporidiosis (Arrowood, 1997).

ii) Using 10% formalin to preserve the animal samples so that they could be imported safely into the UK as described in section 3.1.1.1. This may have affected the stainability and PCR detection of the oocysts, and they were no longer visible in spite of washing before DNA extraction.

iii) Presence of some problems on sample collection such as transport and storage conditions. It has been demonstrated that transport condition are factors which may affect the microscopy result. For example, in a study of water-related samples, storage of oocysts less than 0°C greatly reduced their staining ability by Ap (Smith *et al.*, 1989). However, all samples were kept at -20°C and were transport in ice blocks from the place of the study as described in section 3.1.1.1.

iv) It was difficult to look for diarrhoeal cases among animals and some samples were soft but not frankly diarrhoeal cases. Interestingly, the positive sample that was identified among the sheep was diarrhoeic.

For the animal samples zoonotic activity of the parasite may be very low in this area or the number of oocysts may be very low in the samples. The presence of 10^6 oocysts per gram of stool samples has been indicated by Mtambo *et al.* (1992) as necessary for detection; however a lower limit of 50,000 oocysts per gram has been suggested by Morgan *et al.* (1998).

v) Diarrhoea in those animals might be caused by any other diarrhoeal causes rather than *Cryptosporidium* spp.

Stool specimens were collected from 62 asymptomatic children (used as a comparison group) to look for *Cryptosporidium* oocysts using SMB, ZN and ELISA, and all were negative. This is in agreement with the prevalence in the comparison group among most studies from Saudi Arabia and neighbouring countries with the exception of five studies that reported the prevalence in negative controls of between 1.1% to 4.7% (Nimri & Hijazi, 1994; Mahdi & Ali, 2002b; Mahdi & Ali, 2002a; Al-Braiken *et al.*, 2003; Mahdi & Ali, 2004) (Table 2.1).

The main objective of this chapter was screening of samples to detect *Cryptosporidium* spp. for further investigation on genotyping the parasite.
Direct microscopy of the staining is cost effective and works best when large numbers of oocysts are present in the samples, but is less sensitive in detecting low numbers of oocysts in asymptomatic infections. Therefore, the ELISA technique was performed on all stool samples in the study to confirm the ZN and SMB techniques which were performed on the Gizan samples only. Subsequently, stool samples from Maddina were only screened by ELISA.

The ELISA technique has been developed for screening of large numbers of samples for the presence of *Cryptosporidium* antigens. There are many reports which have indicated that immunodiagnostic methods such as ELISA are more sensitive than microscopy methods (Dagan *et al.*, 1995; Arrowood, 1997; Doing *et al.*, 1999). The ability to use specimens which have been preserved in different preservatives and fixatives such as 10% formalin, methiolate formaldehyde (MF) or sodium acetate, acetic acid and formaldehyde (SAF) and 75% ethanol is the main advantage of the ELISA test (Dagan *et al.*, 1995; Kehl *et al.*, 1995; Marshall *et al.*, 1997). Other advantages of ELISA are: the test is simple and easy to perform in a short time (the test time is about 120-130 minutes per plate for 96 samples) and it is very easy to read the results even in the absence of a spectrophotometer, samples can be either preserved or fresh, centrifugation is not necessary and it is useful for batch testing (Dagan *et al.*, 1995; Kehl *et al.*, 1995; Marshall *et al.*, 1997). Furthermore, the ELISA can detect different *Cryptosporidium* species as shown in the current study.

In the Gizan study, *Cryptosporidium* specific antigen (CSA) was identified in 45/454(9.9%) of samples using the ELISA method (Table 4.1.). The test was positive for 19 samples which were negative and 26 samples which were positive by the staining methods. This indicated that ELISA was more sensitive in detecting *Cryptosporidium* oocysts ($p=0.026$) and the strength of agreement between these two methods was substantial (Kappa value=0.71) (Table 4.3). These results are in agreement with findings obtained from previous studies, (Siddons *et al.*, 1992; McCluskey *et al.*, 1995; el-Shazly *et al.*, 2002; Cirak & Bauer, 2004) but are not in agreement with others, (Kehl *et al.*, 1995; Graczyk *et al.*, 1996; Ignatius *et al.*, 1997; Lindo *et al.*, 1998; Johnston *et al.*, 2003) It has been predicted that all stool samples with $\geq 10^6$ oocysts per ml should yield positive results in a faecal enzyme immunoassay (ProSpecT, Alexon) (Arrowood, 1997; Johnston *et al.*, 2003).

The detection rate of *Cryptosporidium* oocysts was higher by PCR compared to microscopy. The results of available comparative studies of microscopy and PCR methods showed that PCR recognizes significantly more frequently *Cryptosporidium*

infection than microscopy, (Balatbat *et al.*, 1996; Webster *et al.*, 1996; Morgan *et al.*, 1998; McLauchlin *et al.*, 1999; el-Shazly *et al.*, 2002; McLauchlin *et al.*, 2003; Amar *et al.*, 2004; Helmy *et al.*, 2004) but other studies have shown that there is no significant difference between the two techniques (Majewska *et al.*, 2000; Menon *et al.*, 2001).

A total of 101 samples were 18S rRNA PCR positive among 104 samples positive by ELISA. The other three samples negative using 18S rRNA as the target were positive using other molecular targets for the PCR. Previous comparisons of ELISA and PCR methods have shown that they have similar sensitivity and specificity. For example, results obtained from a study in Germany showed that the PCR assay did not increase the detection rate of *Cryptosporidium* infections and the ELISA test is sufficient for routine screening of faecal samples (Bialek *et al.*, 2002). However, el-Shazly *et al.* (2002) found that the sensitivity, specificity and accuracy of ELISA detection of *Cryptosporidium* in relation to detection of DNA in stool by PCR were 84.2%, 96% and 88.8%, respectively, showing an advantage of PCR.

Surprisingly, very low absorbances (0.06 and 0.087) were observed for samples No. 36 and 84 from Samtah and Al-Ardah respectively which were both PCR positive. This demonstrates the high sensitivity of the ELISA technique. The results of this study have also shown that SMB is more sensitive than ZN and it should be used together with ELISA test for screening stool samples.

The detection rate of *Cryptosporidium* positive human samples in Gizan was nearly double (9.9%) the detection rate in Maddina (5.2%). The reported prevalence rates of *Cryptosporidium* spp. among normal human hosts differ from 0.1-2% in developed countries to 0.5-10% in developing countries, in both diarrhoeic and asymptomatic children. However, sporadic reports have demonstrated high prevalence levels of infection at over 30% in some studies (Casemore, 1990). Previously published studies on *Cryptosporidium* in Saudi Arabia have reported the prevalences in different patients ranging between 1% (Khan *et al.*, 1988) and 32% (Al-Braiken *et al.*, 2003). The high prevalence in Gizan compared to Maddina might be due to following two reasons:

i) The climate in Gizan is hot and wet during the summer months (April to October) and warm during the winter months (November to March), while in Madddina it is hot and dry for most of the year extending from April to October, followed by a cool, dry season between December to February.

The seasonal distribution and climatic conditions are risk factors in relationship to the epidemiology of cryptosporidiosis. Several factors could account for seasonal variations in the occurrence of cryptosporidiosis, including factors affecting the numbers 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 (Javier Enriquez *et al.*, 1997; Katsumata *et al.*, 1998).

ii) Gizan is situated in the south west of the kingdom along the Yemen border, where a large number of persons and animals cross between the two countries. The hygiene in Yemen is not as good as in Saudi Arabia and travel is a risk factor for acquiring infection with *Cryptosporidium* spp. Few studies have been performed to assess the prevalence of cryptosporidiosis in long-term travellers. A survey of Peace Corps volunteers who were residing in West Africa demonstrated a 13% increase in sero-prevalence during a 2-year period (Ungar *et al.*, 1989).

The highest incidence of *Cryptosporidium* infection in the Gizan area was in Al-Ardah (20%) and this might be due to the following reasons:

i) It is situated in the south east of Gizan area in close proximity to the Yemen border and the effect of this factor has been explained above.

ii) The low socioeconomic standards of this area compared to the capital city Gizan. *Cryptosporidium* infection (47%) has been found to be the one of most common infections and the majority of patients were of lower socioeconomic status. (Singh *et al.*, 2003). The prevalence of *Cryptosporidium* infection in

children along the Texas-Mexico border has been associated with some risk factors such as lower household income and socioeconomic status (Leach *et al.*, 2000).

iii) The source of water supply in Al-Ardah is service rainwater, which might be an important vehicle responsible for the transmission of the disease. *Cryptosporidium* spp. have been responsible for eight waterborne disease outbreaks associated with water intended for drinking in the United States (Moore *et al.*, 1993; Berkelman, 1994). *Cryptosporidium* spp. accounted for the greatest number of individuals becoming ill from water in which the aetiologic agent of the outbreak could be identified.

iv) The majority of the samples were collected from young children and the mean age of the cases in Al-Ardah was 36.7 months with a range between 1 to 840 months. It had been reported that in developing countries, *Cryptosporidium* infections occur mostly in children younger than 5 years where the peak of infections and diarrhoea appear in children under 2 years old (Tumwine *et al.*, 2003; Steinberg *et al.*, 2004).

The mean age of the positive cases was 19.9 and 19.3 months in Gizan and Maddina respectively. *Cryptosporidium* infection was more common among children less than two years of age in Gizan ($p<0.01$) and Maddina ($P=0.07$) when the detection rate of infection in children less than two years old of age was compared with the detection in children older than two years, this demonstrated the significant relationship between age and *Cryptosporidium* infection. The highest prevalence rates in symptomatic patients were in patients (0-5) months of age (48%) and in patients (6-11) months (11%) in Gizan and Maddina respectively (Table 4.5 and 4.9).

This agrees with a seroepidemiological study of *Cryptosporidium* infection that suggested a common age for infection is in two to three year-old individuals, in two Latin American populations (Ungar *et al.*, 1988) also, the prevalence of Cryptosporidiosis was

highest in children less than two years of age in two different studies from Saudi Arabia (Khan *et al.*, 1988) and Jordan (Youssef *et al.*, 2000).

The maximum prevalence of *Cryptosporidium* infection was during the cooler months in both Gizan and Maddina and the peaks of infection were in January and March respectively. In Gizan, 13% of the diarrhoeal cases that were due to *Cryptosporidium* occurred in January but substantial number of other cases occurred in the whole 5 months period October to February. In contrast, 74% of all positive cases in a year in Maddina occurred in March, with a further 9% in February. In Maddina 74% of all diarrhoeal cases caused by *Cryptosporidium* occurred in March.

The seasonal distribution of cryptosporidiosis varies in different parts of the world (Hart, 1999). Many studies have reported the maximum numbers of cases occurring during January to April which agrees with our results. Of these studies, a study in Australia reported that the majority of cases were between January and March (Puech *et al.*, 2001). Another study from Kuwait reported the maximum numbers of cases, 38 of 51 (75%), were seen during the months January to April, indicating a marked seasonal variation (Iqbal *et al.*, 2001). However temperatures and humidity will be markedly different in these months in the different geographical regions.

In a review on *Cryptosporidium* infections in Saudi Arabia and neighbouring countries by Areeshi *et al* (2007), the seasonality of *Cryptosporidium* varied depending on the geographic locations of the studies but it was generally most prevalent in the rainy season. Interestingly, the peak of cryptosporidiosis in Gizan and Maddina coincide with rain in both cities.

There was a difference between gender in the prevalence of cryptosporidiosis in symptomatic cases in either Gizan or Maddina. Hart (1999) showed that there was no influence of gender in *Cryptosporidium* cases, however two recent reports have concluded that in *Cryptosporidium* infections in children with diarrhoea were influenced by gender (Huang *et al.*, 1998; Puech *et al.*, 2001). Studies among different groups of

immunosuppressed persons from Pakistan indicated that there were more cases in males (87%) among cancer patients and in males (40%) among dialyzed patients while diabetic patients showed no gender differences (Baqai *et al.*, 2005).

The rate of *Cryptosporidium* detection in out-patients (4%) in Maddina was similar to the rate of detection in in-patients (6%) admitted to the hospital. The prevalence of cryptosporidiosis is different for different populations surveyed. This result from Maddina is in agreement with a result obtained by Mercado & Garcia (1995) which concluded that *Cryptosporidium* infections are infrequent in healthy outpatients and that its prevalence was increased in hospitalized children and HIV infected adults. Contrary, Areeshi *et al* (2007) concluded that outpatient studies showed significantly higher prevalences than those where inpatients were included. This may have resulted from the presence of *Cryptosporidium* oocysts in asymptomatic children, some of whom could be considered carriers who act as important reservoirs of the organism and finally a potential source of infection.

5. MULTI-LOCUS GENETIC ANALYSIS OF *CRYPTOSPORIDIUM* SPP.

5.1. Introduction

In multi-locus studies of species differentiation of *Cryptosporidium* parasites a number of different genes have been examined. They were useful in studies aimed to understand the host specificity and the molecular epidemiology of the genus. The identification of *Cryptosporidium* parasites in humans and the source of infection has been an important topic of epidemiological investigations (Xiao, 2003).

Several investigators have used DNA sequence analysis to delineate different species within the genus *Cryptosporidium*. This approach contrasts with traditional methods of defining species based on morphological features or host specificity. After the first PCR assay used for diagnosis of *Cryptosporidium* parasites in stool samples designed by Laxer *et al.*, (1991), several genetic loci have been used for taxonomical purposes, including the small subunit ribosomal RNA (18S rRNA) (Xiao *et al.*, 2000) and adjacent internal transcribed spacer 1 (Morgan *et al.*, 1999a), dihydrofolate reductase (Morgan *et al.*, 1999b), heat shock protein 70 (HSP70) (Sulaiman *et al.*, 2000), ß-tubulin (Pedraza-Diaz *et al.*, 2001), *Cryptosporidium* oocysts wall protein (COWP), and thrombospondin-related adhesive protein (TRAP) (Elwin *et al.*, 2001, Spano *et al.*, 1998b). PCR-RFLP techniques have been described for the differentiation of *Cryptosporidium* spp. based on 18S rRNA (Kimbell *et al.*, 1999, Leng *et al.*, 1996, Lowery *et al.*, 2000). PCR-RFLP based on 18S rRNA was capable of differentiating between *C. parvum* and other *Cryptosporidium* species and it has been the most popular method for the characterization of the parasite (Xiao *et al.*, 2000).

Although RFLP methods are relatively simple, their application is limited by the lack of resolution. The fact that isolates obtained from distant geographical locations and different hosts generated identical RFLP patterns (Spano *et al.*, 1998a) hinted at the need for genotyping methods capable of discriminating among individual isolates, even those belonging to the same subgroup. Direct sequence analysis of various loci has

demonstrated that sequence heterogeneity could be used to differentiate between different isolates (Widmer *et al.*, 1998). More recently, microsatellite length polymorphisms have also been successfully applied (Caccio *et al.*, 2000, Feng *et al.*, 2000).

In this chapter, 18S rRNA PCR and its RFLP were carried out to identify *Cryptosporidium* species which were obtained from screening (Chapter 4). Subsequently, sub-genotyping of *Cryptosporidium* spp. was performed based on the GP60 and HSP70 gene fragments.

5.2. Results

5.2.1. Nested PCR and RFLP for 18S rRNA gene

5.2.1.1. Nested PCR for the 18S rRNA gene

All 104 faecal samples (46 from Gizan and 58 from Maddina) which were found to be positive for *Cryptosporidium* oocysts using microscopy and ELISA methods were submitted for PCR, using the 18S rRNA gene as the target gene. This collection included, 21 positive samples identified by the ZN, SMB staining techniques and ELISA, 5 samples which were identified using the SMB staining technique and ELISA, one sheep sample which was identified using ZN staining technique and ELISA and 77 positive samples that were identified by ELISA only. Few random negative samples by all the 3 screening methods, ZN, SMB and ELISA were submitted for 18S rRNA gene PCR and they were also negative by this method. For the 18S rRNA gene, a nested PCR was carried out using the primer sets AL 1687/1691 and AL 3032/1598, which amplify 1.3 and 0.83 kb segments, respectively, as described previously in section 3.3.2.1. The secondary PCR yielded amplicons of sizes between 834 to 864 bp depending on the *Cryptosporidium* species (Tables 5.1 & Figure 5.1).

Table 5.2 shows the results obtained from PCR of positive samples from different locations in Gizan and Maddina. The samples were tested by targeting the18S rRNA gene and 101/104 (97%) samples yielded PCR products. The three samples which were negative by PCR for 18S rRNA gene were all positive by ELISA alone except for one

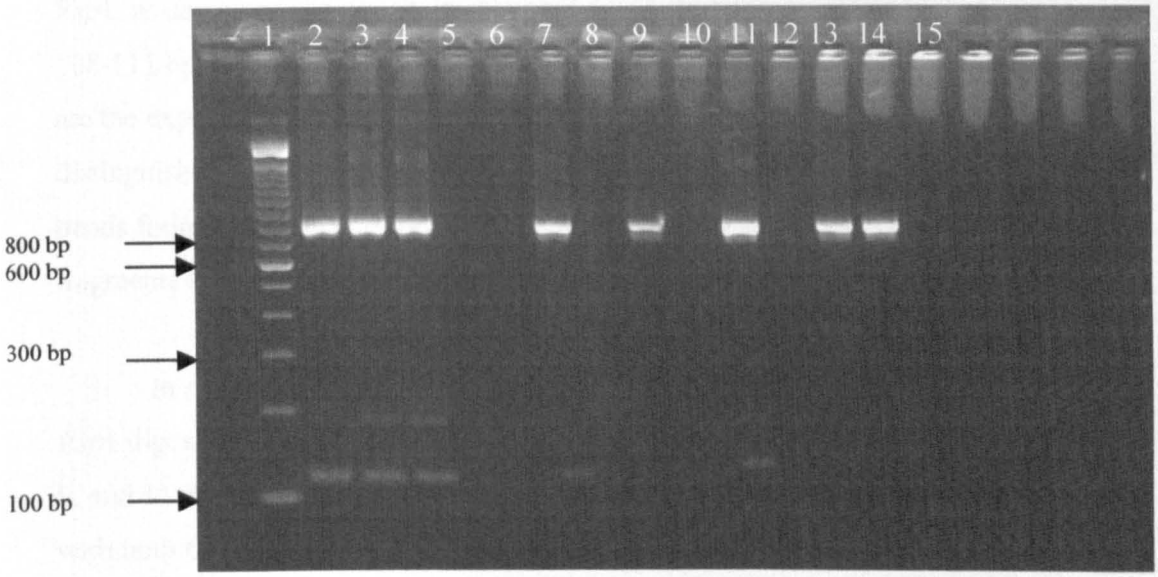


Figure 5.1 The secondary PCR products for 18S rRNA gene of *Cryptosporidium* spp. of approximately 830 bp. Lanes 5,6,8,10 &12 are negative samples and lanes 2-4, 7,9, 11,13 are positive samples used in the current study. The samples are from Al-Ardah, Gizan.

Lanes are:

1: Marker (1 kb)
2: Ar.2
3: Ar.3
4: Ar.12
5: Ar.28
6: Ar.37
7: Ar.40
8: Ar.47
9: Ar.49
10: Ar.54
11: Ar.55
12: Ar.57
13: Ar.58
14: Positive control (D24)
15: Negative control

sample from Al-Ardah (sample no 115) which was also positive by microscopy and later proved to be positive by PCR of GP60 gene.

5.2.1.2. RFLP for 18S rRNA gene

To differentiate between *Cryptosporidium* species, 101 secondary PCR products of the 18S rRNA gene were then digested using two different endonucleases; *Ssp*1 and *Vsp*1. as described previously in section 3.3.3. Following digestion by *Ssp*1, 3 bands of 108-111 bp, 254 bp and 449 bp (Figure 5.2 panel A lanes) were seen. These fragments are the expected sizes for *C. hominis* and *C. parvum* (Table 5.1), which can not be distinguished by this methodology. Only one sample from a sheep showed different bands following digestion by *Ssp*1 which were of 426 bp, 390bp (Figure 5.3). These fragments are the expected size for *C. felis*.

In panel B of Figure 5.2, lanes 3, 4, 8, 10 and 12 showed fragments following *Vsp*1 digestion of approximately 104 and 628 bp, corresponding to *C. parvum*. Lanes 5-7, 9, and 11 showed fragments relating to *C. hominis* of 104 and 561 bp. Mixed infection with both *C. parvum* and *C. hominis* are seen in lanes 2 and 13. The identification of isolates based on the PCR-RFLP is summarized in Table 5.2.

79 (78.2 %) *C. parvum*, 13 (12.9 %) *C. hominis*, 1 (1%) *C. felis* and 8 (7.9%) mixed isolates were detected from 101 samples using PCR-RFLP of the 18S rRNA gene. All the positive isolates with PCR amplification of the 18S rRNA gene in the study were subjected to sequence analysis to allow identification of the sub-genotypes of *Cryptosporidium* spp. and to confirm the RFLP results (see chapter 6).

5.2.2. *Cryptosporidium* species identified among Gizan and Maddina samples:

The identification of isolates based on the PCR-RFLP is summarized in Table 5.2. The most frequent species identified in Gizan was *C. parvum* with 24/43 (55.8%) followed by *C. hominis* with 10/43 (23.3%). *C. felis* was identified in one sheep sample 1/43 (2.3%) while 8/43 (18.6%) samples contained a mixture of the two species, *C.*

parvum and *C. hominis* (Table 5.3). The highest percentage of both *C. parvum* 12/24 (50%) and *C. hominis* 5/10 (50%) in Gizan were identified in Al-Ardah followed by Samtah where 5/24 (20.8%) *C. parvum* and 2/10 (20%) *C. hominis* strains were detected. In addition the highest percentage of mixed samples 6/8 (75%) were also identified in Al-Ardah.

A wide diversity of species was recognised among the Abu-Areesh isolates. This included 3/24 (12.5%) *C. parvum*, 1/10 (10%) *C. hominis*, 1/8 (12.5%) mixed sample and one *C. felis* 1/43 (2.3%) alone that was detected in the study. The eight mixed samples showed RFLP fragments of 104, 561, 628 bp. These fragments are most likely to be a mixture of *C. hominis* (104, 561) and *C. parvum* (104, 628).

In Maddina, *C. parvum* was the dominant species identified 55/58 (94.8 %) followed by *C. hominis* 3/58 (5.2 %).

6/8 (75%) of the mixed infections were detected in male patients and 5/8 (62.5%) of the patients with the mixed infections were in their first year of life.

Table 5.1. PCR product size and restriction products based on 18S rRNA gene of *Cryptosporidium* spp. for the samples identified in this study.

Species	Number	PCR fragment size (bp)	*Ssp*1 digestion product size (bp)	*Vsp*1 digestion product size (bp)
C. parvum	79	834	108,254,449	104,628
C. hominis	13	837	111,254,449	70,104,561
C. felis	1	864	15,33,390,426	104,182,476

Table 5.2. **Genotype testing of *Cryptosporidium* spp. of the sample collections using 18S rRNA PCR-RFLP (n=104).**

Location	No.tested	*C. parvum* n (%)	*C. hominis* n (%)	*C. felis* n (%)	Mixed n (%)	Negatives n (%)
Al-Ardah	26	12 (46.2)	5 (19)	0 (0)	6 (23)	3 (11.5)
Samtah	8	5 (62.5)	2 (25)	0 (0)	1 (12.5)	0 (0)
Abu-Areesh	5	3 (60)	1 (20)	0 (0)	1 (20)	0 (0)
Al-Khobah	2	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Gizan City	4	2 (50)	2 (50)	0 (0)	0 (0)	0 (0)
Maddina	58	55 (95)	3 (5)	0 (0)	0 (0)	0 (0)
Abu-Areesh (sheep)	1	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
Total	**104**	**79 (76)**	**13 (12.5)**	**1 (1)**	**8 (7.7)**	**3 (2.9))**

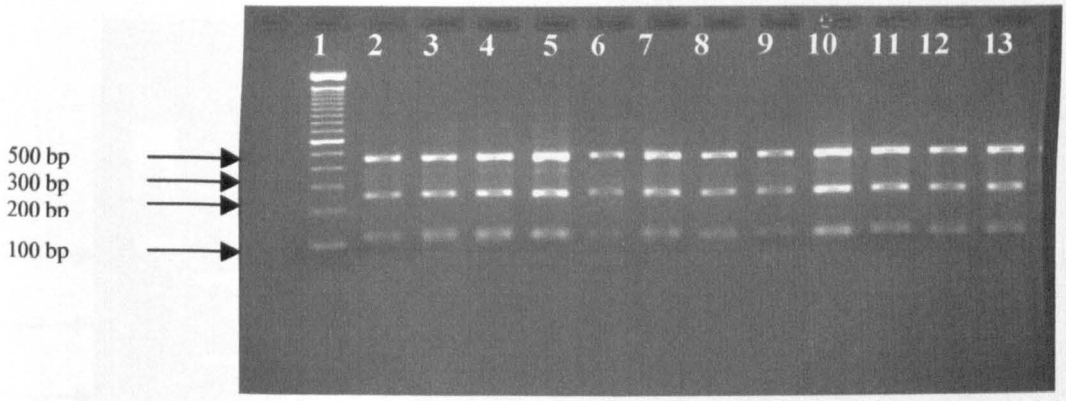
N: Negative PCR.

Table 5.3. **Genotyping testing of mixed *Cryptosporidium* species or genotypes samples of the study using 18S rRNA PCR-RFLP (n=8).**

Sample no.	Source	Patient age (month)	Patient gender	Species identified
3	Al-Ardah	60	M	*C. parvum* + *C. hominis*
12	Al-Ardah	4	M	*C. parvum* + *C. hominis*
64	Al-Ardah	2	F	*C. parvum* + *C. hominis*
73	Al-Ardah	6	M	*C. parvum* + *C. hominis*
101	Al-Ardah	5	M	*C. parvum* + *C. hominis*
130	Al-Ardah	12	F	*C. parvum* + *C. hominis*
2	Samtah	48	M	*C. parvum* + *C. hominis*
28	Abu-Areesh	20	M	*C. parvum* + *C. hominis*

M=Male. F=Female

Panel (A)



Panel (B)

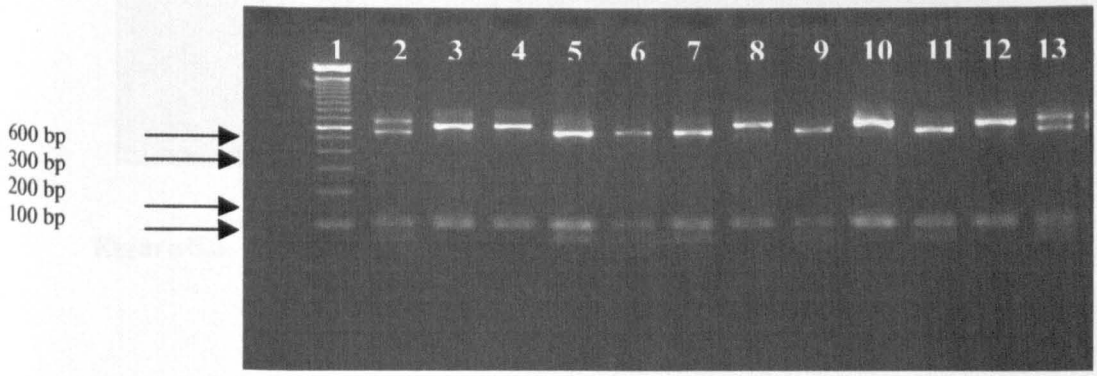


Figure 5.2. The RFLP for 18S rRNA gene. Panel (A) showing the results of restriction digestion with *Ssp*1. Panel (B) shows the digestion patterns with *Vsp*1, showing bands expected for *C. parvum* (lanes 3,4,8,10, 12), *C. hominis* (lanes 5-7,9,11) and *C. parvum* and *C. hominis* mixed (lane 2 &13). The samples are from Al-Ardah, Al-Khobah and Samtah.

Lanes are:

1: Marker (1kb)
2: Ar 101
3: Ar 102
4: Ar 103
5: Ar 108
6: Ar 110
7: Ar 117
8: Kh 1
9: S 92
10: S 47
11: S 84
12: Ab 5
13: Positive control (Ar 12)

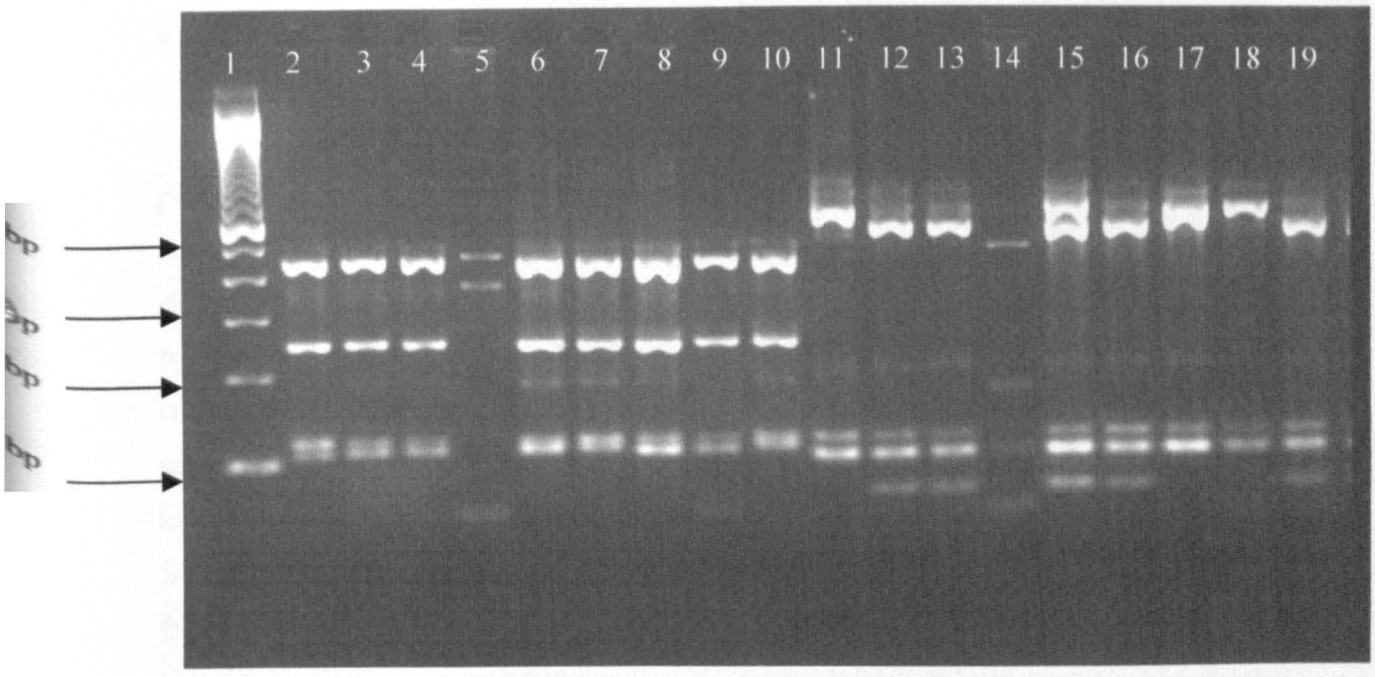


Figure 5.3. The RFLP for 18S rRNA gene by restriction endonuclease digestion of different samples from Gizan Area. Results of restriction digestion with *Ssp*1 are shown through lanes 2-10 while the results of restriction digestion for the same samples with *Vsp*1 are shown through lanes 11-19. Lane 5 & 14 show bands expected for *C. felis*. The digestion patterns with *Vsp*1 show the bands expected for *C. hominis* (lanes 12,13,16,19), *C. parvum* (lanes 11,17,18), *C. parvum* and *C. hominis* mixed (lane 15).

Lanes are:

1: Marker (1kb)
2 & 11: Ab 41
3 & 12: Ab 57
4 & 13: Ar 79
5 & 14: Ab(sheep)
6 & 15: Ar 130
7 & 16: G 5
8 & 17: G 22
9 & 18: G 90
10 & 19: Positive control (G 95)

5.2.3. Nested PCR for the GP60 gene

The GP60 gene was amplified using a nested PCR with primer sets LX001F1 and LX002R1 in the primary PCR and LX003F2 and LX004R2 in the secondary PCR. PCR products of approximately 350-480 bp were produced (Figure 5.4).

Among 104 samples positive by ELISA, 95(91.3%) isolates yielded a PCR product for the GP60 locus. Within these isolates, 42/95 (44.2%) were from the Gizan Area and 53/95 (55.8%) were from Maddina. Among 101 samples positive for the 18S rRNA gene, 92 (91%) isolates yielded a PCR product for the GP60 locus. Three isolates from Al-Ardah (Ar37, Ar84 and Ar115) which did not yield any PCR product for the 18S rRNA gene yielded a PCR product for the GP60 locus. One sheep sample from Gizan which has been identified by 18S rRNA RFLP technique as *C. felis* failed to amplify using the GP60 primer sets. GP60 gene PCR amplified all the ELISA positive isolates from Abu-Areesh, Samtah, Al-Khobah, and Gizan, with 23/26 (88.5%) of the isolates from Al-Ardah and 53/58 (91.4%) of the isolates from Maddina. Few random negative samples by all the 3 screening methods, ZN, SMB and ELISA were submitted for GP60 gene PCR and they were also negative by this method.

All the positive isolates coming of GP60 in the study were subjected to differentiation of allelic group of *C. parvum* and *C. hominis* by sequence analysis (see chapter 6).

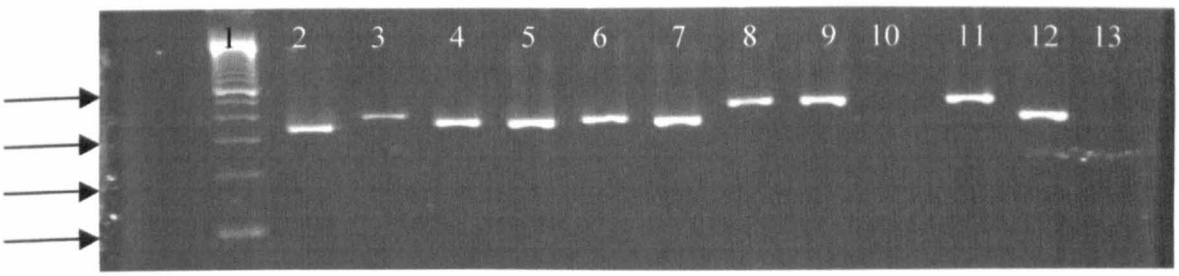


Figure 5.4. The nested PCR of GP60 for *Cryptosporidium* spp. producing approximately 350-480 bp bands. Lanes 10,13 are negative samples. The rest are positive samples. The samples are from Al-Khobah, Samtah and Al-Ardah.

Lines in the upper panel are:

1: Marker (1kb)
2: Kh.5
3: S2
4: S4
5: S6
6: S14
7: Ar2
8: Ar3
9: Ar12
10: Ar28
11: Ar37
12: Positive control (D24)
13: Negative control

5.2.4. Nested PCR for the HSP70 gene

For sub-genotype studies, the HSP70 gene was amplified by nested PCR with primer sets F1 and R1 in the primary PCR and F2 and R2 in the secondary PCR that yielded a PCR product of about 1200 bp (Figure 5.5).

Among 104 samples positive by ELISA, 88 (84.6%) isolates yielded a PCR product for the HSP70 locus. Within these isolates, 38/88 (43.2%) were from the Gizan Area and 50/88 (56.8%) were from Maddina. Among 101 samples positive for the 18S rRNA gene, 87 (86.1%) isolates yielded a PCR product for the HSP70 locus. One isolate from Al-Ardah (Ar84) which did not yield any PCR product for the 18S rRNA gene yielded a PCR product for the HSP70 locus. One sheep sample from Gizan which was identified by 18S rRNA RFLP technique as *C. felis* failed to amplify using the HSP70 primer sets. HSP70 gene PCR amplified all the ELISA positive isolates from Abu-Areesh and Al-Khobah, 7/8 (87.5%) of the isolates from Samtah, 50/58 (86.2%) of the isolates from Maddina, 22/26 (84.6%) of the isolates from Al-Ardah and 2/4 (50%) of the isolates from Gizan. Few random negative samples by all the 3 screening methods, ZN, SMB and ELISA were submitted for HSP70 gene PCR and they were also negative by this method.

All the positive isolates of HSP70 in the study were subjected to sequence analysis to allow identification of the sub-genotypes of *Cryptosporidium* spp. (see chapter 6).

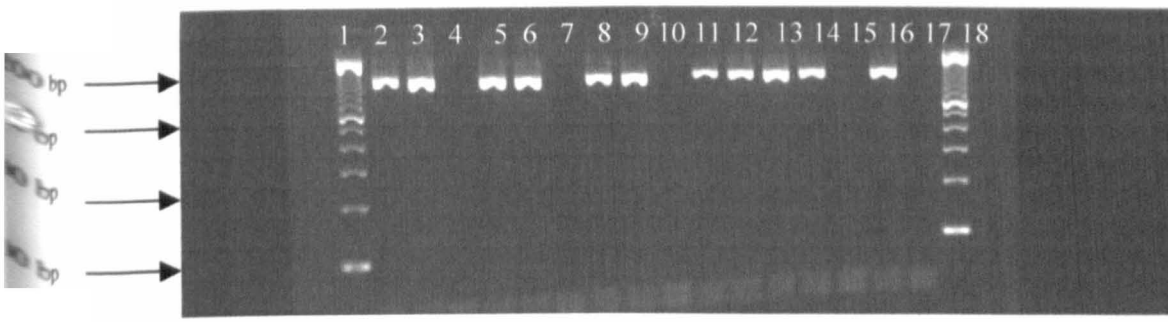


Figure 5.5. The nested PCR of HSP70 gene of *Cryptosporidium* spp. producing bands of approximately 1200 bp. Lanes 4, 7, 10, 15, and 17 shows the negative samples and the rest are positive samples. The samples are from Al-Ardah, Samtah and Maddina collections.

Lanes in the upper panel are:

1 & 18: Marker
2: Md 878
3: Ar12
4: Md 1060
5: Ar73
6: Ar79
7: Ar 110
8: Md 953
9: Md 963
10: Md 1076
11: S 2
12: S 4
13: Md 1113
14: Md 1128
15: Md 1133
16: Positive control (D24)
17: Negative control

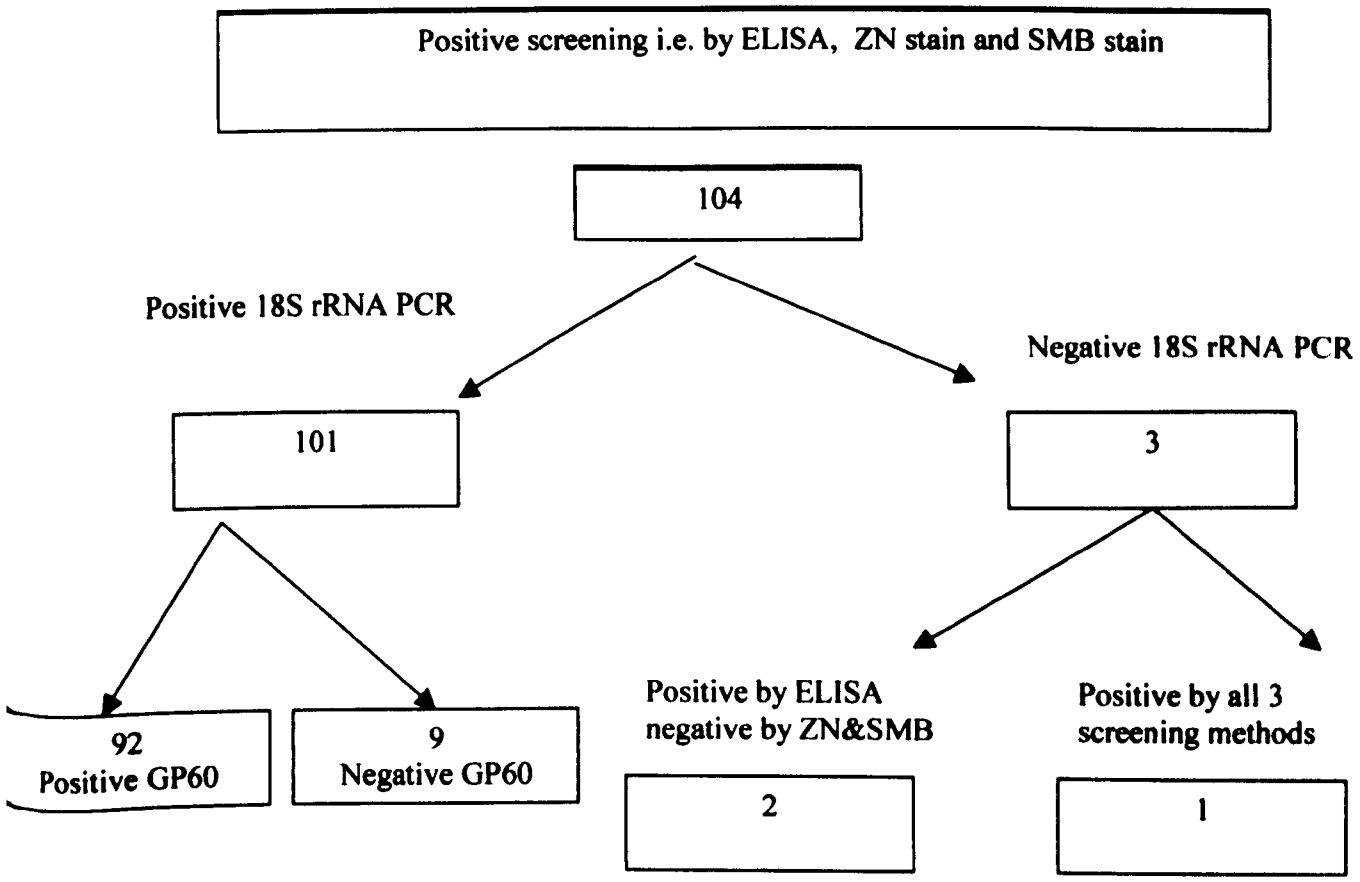
5.3. Discussion

This chapter has described the results of PCR and RFLP of 18S rRNA gene fragments and PCR of GP60 and HSP70 genes of 104 isolates identified by microscopy and ELISA. All the samples positive by screening methods were initially amplified by 18S rRNA-based PCR. Samples that were positive for *Cryptosporidium* by this gene were further genotyped by an 18S rRNA-based PCR-RFLP technique (Xiao *et al.*, 1999). All the positive samples by screening methods were also amplified by PCR of GP60 (Zhou *et al.*, 2003) and HSP70 genes (Gatei *et al.*, 2006) for sub-genotyping.

The faecal samples were stored either at -20 °C or -80 °C without any preservative except for the animal samples which were preserved in 10% Formalin. DNA was extracted using the QIAamp® DNA stool mini kit which had a high rate of *Cryptosporidium* amplification in a simple and consistent procedure (Bialek *et al.*, 2002). The primers which were used for the18S rRNA gene were based on highly conserved regions of the *Cryptosporidium* genus (Xiao *et al.*, 1999) which are widely applicable. The nested PCR protocol that was used in the current study for this gene increased confidence in the confirmation of the identity of the PCR product by successful annealing of the internal nested set of primers.

Of 104 positive samples of *Cryptosporidium* spp. which were obtained by screening methods, 101 samples yielded a PCR product and only 3 samples from Al-Ardah (Ar 37, Ar 84 and Ar 115) failed to produce amplicons for the 18S rRNA gene in spite of repeating the 18S rRNA PCR many times. Two of the 3 negative samples (Ar 37 and Ar 84) were positive only by ELISA and one sample (Ar 115) was positive by both ZN and SMB staining methods and ELISA. Subsequently, all the three negative samples with 18 S rRNA gene yielded a PCR product for the GP60 gene and one sample (Ar 84) yielded a PCR product for the HSP70 gene (Figure5.6).

Figure 5.6. Summary of results of genetic analysis based on the two genes, 18S rRNA, and GP60.



Failure to yield PCR products from these three samples for 18S r RNA gene could be related to the variations in the primer binding region which may occur in novel species and may result in lower specificity and amplification failures. Therefore, the method may require further standardisation for specific species or genotypes because of the sequence polymorphisms in the 18S rRNA locus as suggested by Widmer *et al.* (1998).

However the sequences of GP60 gene of the three negative samples from Al-Ardah by 18S rRNA gene (Ar37, Ar84 and Ar115) showed that they were *C. parvum* while the sequences of HSP70 gene confirmed that Ar84 was *C. parvum* and the PCR of this gene was negative for the other two samples (Ar37 and Ar115).

A useful method for detecting genetic differences in populations without sequencing PCR products involves amplifying a sequence and cutting the product with restriction enzymes to analyse restriction fragment length polymorphism (RFLP) (reviewed by Careno *et al.*, 2001). It has also been proved that the PCR-RFLP of the18S rRNA gene is a useful tool for differentiating *Cryptosporidium* isolates, variations in the gene being useful for the identification of the species of the parasite (Leng *et al.*, 1996, Xiao *et al.*, 1999). It has been possible to distinguish the various *Cryptosporidium* species reliably and to identify novel species in humans and animals using analysis of this segment of the gene (Xiao *et al.*, 1999, Guyot *et al.*, 2001, Gatei *et al.*, 2002, Ong *et al.*, 2002, Matsubayashi *et al.*, 2004). Further confidence in the technique is gained by using two restriction endonucleases (Xiao *et al.*, 1999).

Thus, in this thesis different *Cryptosporidium* species based on PCR-RFLP have been identified using 101 PCR amplicons from the18S rRNA gene which were all amplified by PCR. Table 5.2 shows the results obtained from this study. Among 100 human isolates, 79 (79%) *C. parvum*, 13 (13%) *C. hominis* and 8 (8%) mixed infections of *C. parvum/C. hominis* were identified. Genotyping of isolates derived from humans indicated that infections of both anthroponotic and zoonotic species had occurred. This finding is similar to results obtained in other studies elsewhere (Morgan *et al.*, 2000,

Alves *et al.*, 2001, Guyot *et al.*, 2001, Glaeser *et al.*, 2004). Also, these studies revealed that *C. parvum* and *C. hominis* are the major isolates which were detected by RFLP of 18S rRNA gene. However, in some studies the number of *C. parvum* cases is greater than those with *C. hominis* (Morgan *et al.*, 2000, Alves *et al.*, 2001, Guyot *et al.*, 2001) but in other studies *C. hominis* was more prevalent (Enemark *et al.*, 2002, Gatei *et al.*, 2003, Glaeser *et al.*, 2004). The majority of isolates detected in this work were *C. parvum* which is a potentially zoonotic species.

On the basis of our knowledge, this is the first genotyping study from Gizan and Maddina, Saudi Arabia. The isolates from Al-Ardah exhibited considerable diversity. The isolates included 12/23 (52.2%) *C. parvum*, 5/23 (21.7%) *C. hominis* and 6/23 (26%) mixed infections. Detection of *C. parvum* as the most common species in the Al-Ardah collection is similar to findings obtained from other studies in the Middle-East (Al-Braiken *et al.*, 2003, Sulaiman *et al.*, 2005).

Among 58 human isolates from Maddina, 55 (95%) and 3 (5%) were identified as *C. parvum* and *C. hominis*, respectively. This finding is similar to the studies of McLauchlin *et al.*(2000) and Gatei *et al.* (2003) and in contrast to those of others (Glaeser *et al.*, 2004, Llorente *et al.*, 2007). Nevertheless it is difficult to compare human cryptosporidial infections from the data available because the examined populations, as well as the techniques used to detect infection, vary between studies and comparative data are not readily available. AIDS and other conditions of immune suppression increase the infection rate of cryptosporidiosis and the range of parasite species involved (Baqai *et al.*, 2005). For instance in the UK, travel history in cases of *C. hominis* and contact to farm animals in cases of *C. parvum* were found to be important (McLauchlin *et al.*, 2000, Gasser *et al.*, 2003).

Among the animal samples, *C. felis* was the only species detected from a sheep in an animal farm in Abu-Areesh. This finding is in agreement with the results obtained by Werner *et al.* (2004) in a study to evaluate the usefulness of different methods for

detection of *Cryptosporidium* in human and animal stool samples. They concluded that *C. felis* could infect different animal species including sheep, horses, cats and dogs,.(Werner *et al.*, 2004).

18S rRNA-RFLP detected 8 mixed *Cryptosporidium* species samples in the study. This included 6 samples from Al-Ardah, one sample from Samtah and one sample from Abu-Areesh. In each of these mixed samples the two *Cryptosporidium* species identified were *C. parvum* and *C. homins*. The majority of the mixed cases occurred in male children 6/8 (75%) and in the age group between 2 and 12 months (62.5 %). Donna *et al.*, (2003) have suggested that when the two species (*C. hominis* and *C. parvum*) simultaneously infect the same host, *C. parvum* invariably predominates, displacing *C. hominis* within a short period of time.

The next part of this project involved PCR of a highly polymorphic single copy gene, GP60, which encodes a glycoprotein that subdivides *C. hominis* and *C. parvum* into several allelic groups. Each group consists of multiple subtypes (Strong *et al.*, 2000, Peng *et al.*, 2001, Sulaiman *et al.*, 2001, Glaberman *et al.*, 2001, Leav *et al.*, 2002).

Nested PCR for the GP60 gene was carried out on 104 samples which were positive by screening methods. A total of 95 (91.3%) samples yielded a PCR product for this gene and the rest, including the one isolate of *C. felis* did not yield any amplicons. Failure to yield a PCR product for the GP60 gene for some samples was not surprising because similar results have been obtained by other investigator (Peng *et al.*, 2003). The failure to amplify PCR products from this gene may happen because there is only a single copy of the gene, with larger amplicons and possible primer mismatches (Alves *et al.*, 2003). Failure of the *C. felis* isolate may be due to DNA degradation of the sample because 18S rRNA PCR and even DNA extraction was repeated for this sample but negative results were obtained. All the mixed species samples yielded PCR products for the GP60 gene. All the positive GP60 PCR samples were sent for sequence analysis. The

findings and sub-genotype details of these isolates will be presented in the following chapter.

The last part of this chapter was performed to investigate possible variation within species using the HSP70 gene. This gene encodes a heat shock protein which belongs to a multi-gene family that is a good target for genotyping, due to its high level of heterogeneity spread over the entire sequence (Sulaiman *et al.*, 2000).

Nested PCR for the HSP70 gene was carried out for 104 samples which were positive by screening methods. Only 88 (84.6%) isolates yielded a PCR product for this gene and the rest, including the one isolate of *C. felis* did not yield any amplification product. The reason for the failure of some isolates to amplify PCR products of this gene could be as explained previously for GP60 (Sulaiman *et al.*, 2001). Each of the mixed species samples yielded PCR products for HSP70 gene. All the positive HSP70 PCR samples were sent for sequence analysis. The sub-genotype details of these isolates will be discussed in chapter 6.

In conclusion, this study has shown that the 18S rRNA gene has some advantages for identification of the majority of samples. Therefore, the application of multi-locus gene analysis should help to determine the level of intra-specific variation in the *Cryptosporidium* genus. However, further conformation to identity of those isolates is crucial using sequence analysis which will be described in the next chapter.

6. NUCLEOTIDE SEQUENCING AND PHYLOGENY

6.1 Introduction

Molecular tools developed to detect and differentiate the species/genotypes and subtypes of parasites have become useful in the study of population structure and transmission dynamics of parasites (Xiao & Ryan, 2004). Comparison of DNA or protein sequences is the basis of molecular phylogenetics and the science of estimating evolutionary relationships (Baldauf, 2003).

To understand the phylogeny of the genus *Cryptosporidium*, particularly with respect to closely related genotypes of these parasites, a multi-locus approach is needed. The sequences available for *Cryptosporidium* parasites are those such as 18S rRNA (Xiao *et al.*, 1999a; Xiao *et al.*, 1999b) and HSP70 (Sulaiman *et al.*, 2000). Furthermore, these sequences represent different types of genes in the *Cryptosporidium* genome evolving at different speeds. There is a multi-copy ribosomal RNA gene (18S rRNA) and a single-copy gene coding for a functional protein (HSP70) (Le Blancq *et al.*, 1997; Spano & Crisanti, 2000). These characteristics make 18S rRNA and HSP70 genes of *Cryptosporidium* useful targets in *Cryptosporidium* genotyping, characterization and phylogenetic studies.

Subsequently, cloning and sequencing of several alleles of the highly polymorphic single-copy *C. hominis* and *C. parvum* GP60 gene have demonstrated their unprecedented degree of sequence polymorphism, which is far greater than that observed for any other gene or protein studied in these parasites (Leav *et al.*, 2002). Members of different allele families differ from each other extensively in their primary sequence, but within each family, sub-genotypes differ from each other mostly in the numbers of tri-nucleotide repeats (Xiao, 2003).

This chapter describes the results of sequencing of the secondary PCR products of all the positive isolates from different regions in Gizan and Maddina using 18S rRNA,

GP60 and HSP70 genes in order to assess the diversity and phylogeny of Cryptosporidia from the different regions. This chapter also describes the results of sequencing the clones of the 8 mixed samples which were previously shown likely to be mixed infections based on PCR-RFLP of the 18S rRNA gene.

6.2. Results

6.2.1. Sequence identity

All the positive samples which were identified by the screening studies from Gizan and Maddina were sequenced at three loci (18S rRNA, GP60 and HSP70) and the sequences used for comparison with the GenBank database. Table 3.7 summarizes the GenBank accession numbers of sequences used for sequence comparison. Also, Table 6.1 shows the results of species identification for all isolates with the exception of the 8 mixed isolates based on the different loci examined. In this table, the content of species column is based on PCR-RFLP of the 18S rRNA gene of the isolates.

Table 6.1. The results of species identified based on the different loci examined (n=96).

Isolate	Source	Species	18S rRNA	GP60	HSP70
Ar2	Al-Ardah	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Ar37	Al-Ardah	Unknown	Neg. PCR	*C. parvum*	Neg. PCR
Ar40	Al-Ardah	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Ar49	Al-Ardah	*C. parvum*	*C. parvum*	Neg. PCR	Neg. PCR
Ar55	Al-Ardah	*C. hominis*	*C. hominis*	*C. hominis*	*C. hominis*
Ar58	Al-Ardah	*C. parvum*	*C. parvum*	Neg. PCR	*C. parvum*
Ar63	Al-Ardah	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Ar74	Al-Ardah	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Ar79	Al-Ardah	*C. hominis*	*C. hominis*	*C. hominis*	*C. hominis*
Ar82	Al-Ardah	*C. parvum*	*C. parvum*	Sequence failed	*C. parvum*
Ar84	Al-Ardah	Unknown	Neg. PCR	*C. parvum*	*C. parvum*
Ar87	Al-Ardah	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Ar92	Al-Ardah	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Ar94	Al-Ardah	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Ar102	Al-Ardah	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Ar103	Al-Ardah	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Ar108	Al-Ardah	*C. hominis*	*C. hominis*	*C. hominis*	*C. hominis*
Ar110	Al-Ardah	*C. hominis*	*C. hominis*	Neg. PCR	Neg. PCR
Ar115	Al-Ardah	Unknown	Neg. PCR	*C. parvum*	Neg. PCR
Ar117	Al-Ardah	*C. hominis*	*C. hominis*	*C. hominis*	*C. hominis*
S4	Samtah	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
S6	Samtah	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
S14	Samtah	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
S36	Samtah	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
S47	Samtah	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
S84	Samtah	*C. hominis*	*C. hominis*	*C. hominis*	*C. hominis*
S92	Samtah	*C. hominis*	*C. hominis*	Sequence failed	Neg. PCR
Ab5	Abu-Areesh	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Ab41	Abu-Areesh	*C. parvum*	*C. parvum*	*C. parvum*	Sequence failed
Ab57	Abu-Areesh	*C. hominis*	*C. hominis*	*C. hominis*	*C. hominis*
Ab64	Abu-Areesh	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Kh1	Al-Khobah	*C. parvum*	*C. parvum*	Sequence failed	*C. parvum*
Kh5	Al-Khobah	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
G5	Gizan City	*C. hominis*	*C. hominis*	Sequence failed	Sequence failed
G22	Gizan City	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
G90	Gizan City	*C. parvum*	*C. parvum*	*C. parvum*	Neg. PCR
G95	Gizan City	*C. hominis*	*C. hominis*	Sequence failed	Neg. PCR
Md18	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md125	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md126	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	Neg. PCR

Isolate	Source	Species	18S rRNA	GP60	HSP70
Md154	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md393	Maddina	*C. hominis*	*C. hominis*	Neg. PCR	Neg. PCR
Md408	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md553	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md644	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md647	Maddina	*C. parvum*	*C. parvum*	Neg. PCR	Neg. PCR
Md769	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md782	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md790	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1180	Maddina	*C. hominis*	*C. hominis*	*C. hominis*	Neg. PCR
Md1187	Maddina	*C. parvum*	*C. parvum*	Sequence failed	*C. parvum*
Md878	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md879	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md897	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md915	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	Sequence failed
Md917	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md953	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md954	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md957	Maddina	*C. parvum*	*C. parvum*	Sequence failed	*C. parvum*
Md963	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md968	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md969	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md987	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1021	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1024	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	Sequence failed
Md1029	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1030	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1034	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1038	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1043	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1047	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1049	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1055	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1060	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	Neg. PCR
Md1061	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1068	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1071	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1072	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1076	Maddina	*C. parvum*	*C. parvum*	Neg. PCR	Neg. PCR
Md1103	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1106	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1107	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1108	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*

Isolate	Source	Species	18S rRNA	GP60	HSP70
Md1113	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1127	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	Sequence failed
Md1128	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1132	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1133	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	Neg. PCR
Md1143	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1146	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1157	Maddina	*C. parvum*	*C. parvum*	Neg. PCR	*C. parvum*
Md1163	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	*C. parvum*
Md1167	Maddina	*C. parvum*	*C. parvum*	*C. parvum*	Sequence failed
Md1178	Maddina	*C. hominis*	*C. hominis*	Neg. PCR	Neg. PCR
Sheep61	Abu-Areesh	*C. felis*	x	Neg. PCR	Neg. PCR

X= No sequence was obtained for this sample

6.2.1.1. The 18S rRNA gene

Partial sequences from the 18S rRNA gene were obtained from *C. parvum* (79 isolates), and *C. hominis* (13 isolates). The lengths of the gene fragments varied from 834-837 bp depending on the species. The gene fragments of *C. hominis* isolates were the longest and those of *C. parvum* were the shortest.

A BLAST search (NCBI) of the sequences confirmed their identity which was in agreement with the results of the species identifications obtained by PCR-RFLP analysis of all isolates of *Cryptosporidium* spp.

Each of the 13 *C. hominis* isolates from both Gizan and Maddina showed 98%-100% identity to the published isolate CHZF2 (Accession number EF570922) in GeneBank.

Each of the 79 *C. parvum* strains from both Gizan and Maddina, showed 98%-100% sequence identity to the published strain of *C. parvum* Izatnagar (Accession number EF611871).

6.2.1.2. The GP60 gene

Sequencing of the GP60 gene was also achieved for *C. parvum* (77 samples) and *C. hominis* (10 samples). Importantly, sequence analysis of the GP60 gene divides the species *C. hominis* and *C. parvum* into at least eight allele families, each of which has several genotypes. Ia, Ib, Id, Ie and If are *C. hominis* allele families and IIa, IIb, IId and IIc (previously known as Ic) are *C. parvum* allele families. (Strong *et al.*, 2000; Alves *et al.*, 2003; Xiao, 2003).

Among the *C. hominis* isolates, 6 isolates from Gizan (4 from Al-Ardah, one from Samtah and another one from Abo-Areesh) with allele group Ie subtype IeA11G3T3

showed 99%-100% sequence identity to the published strain of 4302 (Accession number DQ665696). One isolate from Maddina with allele group Ib had 98% similarity to the published strain of A48 (Accession number AY382672).

Among the *C. parvum* isolates, 40 isolates with allele group IId (28 isolates from Maddina , 7 isolates from Al-Ardah, 3 isolates from Samtah and one isolate each from Al-Khobah and Gizan City) showed 97%-100% sequence identity to the published strain of 5635 (Accession number AY738186).

18 isolates with allele group IId subtype IIdA22G1 (9 isolates from Maddina, 3 isolates from Al-Ardah, 3 isolates from Abo-Areesh, 2 isolates from Samtah and one isolate from Gizan city) showed 97%-100% sequence identity to the published strain of SH75 (Accession number EF025582).

11 isolates with allele group IId (10 isolates from Maddina and one isolate from Al-Ardah) showed 96%-100% sequence identity to the published strain of 8897 (Accession number AY738194).

3 isolates from Maddina with allele groups IIa subtype IIaA14G2R1b, IIc subtype IIcA5G3a and IId subtype IIdA20G1a showed 99% sequence identity to the published strains of 8953, 8902 and 8025 (Accession numbers DQ871350, AY738195 and AY738193) respectively.

One isolate from Al-Ardah with allele group IIc subtype IIcA5G3 99% sequence identity to the published strain of H14 (Accession number EF025581).

The sequence for the GP60 locus failed with some samples, although these samples had been successfully sequenced for the 18S rRNA locus.
The allele families, *C. parvum* IIb and *C. hominis* Ia, Id and If were not found in the present study (Table 6.2).

Table 6.2. ***Cryptosporidium*** **allele groups identified based on GP60 gene locus**

No. of isolates	Species and allele group	Reference strain	Subtype	Accession number
6	*C. hominis* Ie	4302	IeA11G3T3	DQ665696
1	*C. hominis* Ib	A48	Unknown	AY382672
40	C. parvum IId	5635	IIdA20G1d	AY738186
18	C. parvum IId	SH75	IIdA22G1	EF025582
11	C. parvum IId	8897	IIdA18G1	AY738194
1	C. parvum IIa	8953	IIaA14G2R1b	DQ871350
1	C. parvum IIc	8902	IIcA5G3a	AY738195
1	C. parvum IId	8025	IIdA20G1a	AY738193
1	C. parvum IIc	H14	IIcA5G3	EF025581

6.2.1.3. The HSP70 gene

Partial sequences from the HSP70 gene were obtained from *C. hominis* (7 isolates) and *C. parvum* (73 isolates). All the *C. parvum* isolates showed 98%-100% sequence identity to the published strain of Iowa II (Accession number XM_625373). Among the *C. hominis* isolates, 4 isolates from Al-Ardah, one isolate from Samtah and another one from Abo-Areesh showed 98%-99% sequence identity to the published strain of CHZF2 (Accession number EF591788).

The sequence for the HSP70 locus failed with a few samples even though these samples had been successfully sequenced for the 18S rRNA locus.

6.2.2. Phylogeny

The sequences of two isolates, Ar108 from Gizan which was identified as *C. hominis* and Md1055 from Maddina which was identified as *C. parvum*, were used to compare the identities and relationship between the three genes 18S rRNA, GP60 and HSP70 and to evaluate the distances from the published strains in the GeneBank. These two strains were chosen specifically because their gene sequencing was successful and because they represent the most commonly detected strains in the study. Different published strain sequences were used from the GeneBank including some of the most common reference strains with the sequences of the two samples chosen of our study to draw the phylogenetic relationship among these isolates based on the Neighbour-Joining (NJ) phylogram method.

6.2.2.1. The phylogeny of the 18S rRNA gene

C. muris mouse genotype IDRH-13 was used from the GeneBank as the out-group as this species showed least similarity to the others (Figure 6.1). This species formed a separate clade with full statistical reliability (100%) with another clade that consisted of

C. hominis and *C. parvum*. Our first sample Ar108 which was identified as *C. hominis* is in one group with its reference strain from the GeneBank *C. hominis* isolate CHZF2 (Accession number EF570922) and they are supported as a group by a bootstrap value of 96%. Our second sample Md1055 that was identified as *C. parvum* is in one group with its reference strain from the Gene Bank *C. parvum* isolate Izatnagar (Accession number EF611871) and they are supported as a group by a bootstrap value of 95%.

Both of the two samples Md1055 (*C. parvum*) and Ar108 (*C. hominis*) partial 18S rRNA gene sequences have been deposited in GenBank under accession numbers AB369993 and AB369994 respectively.

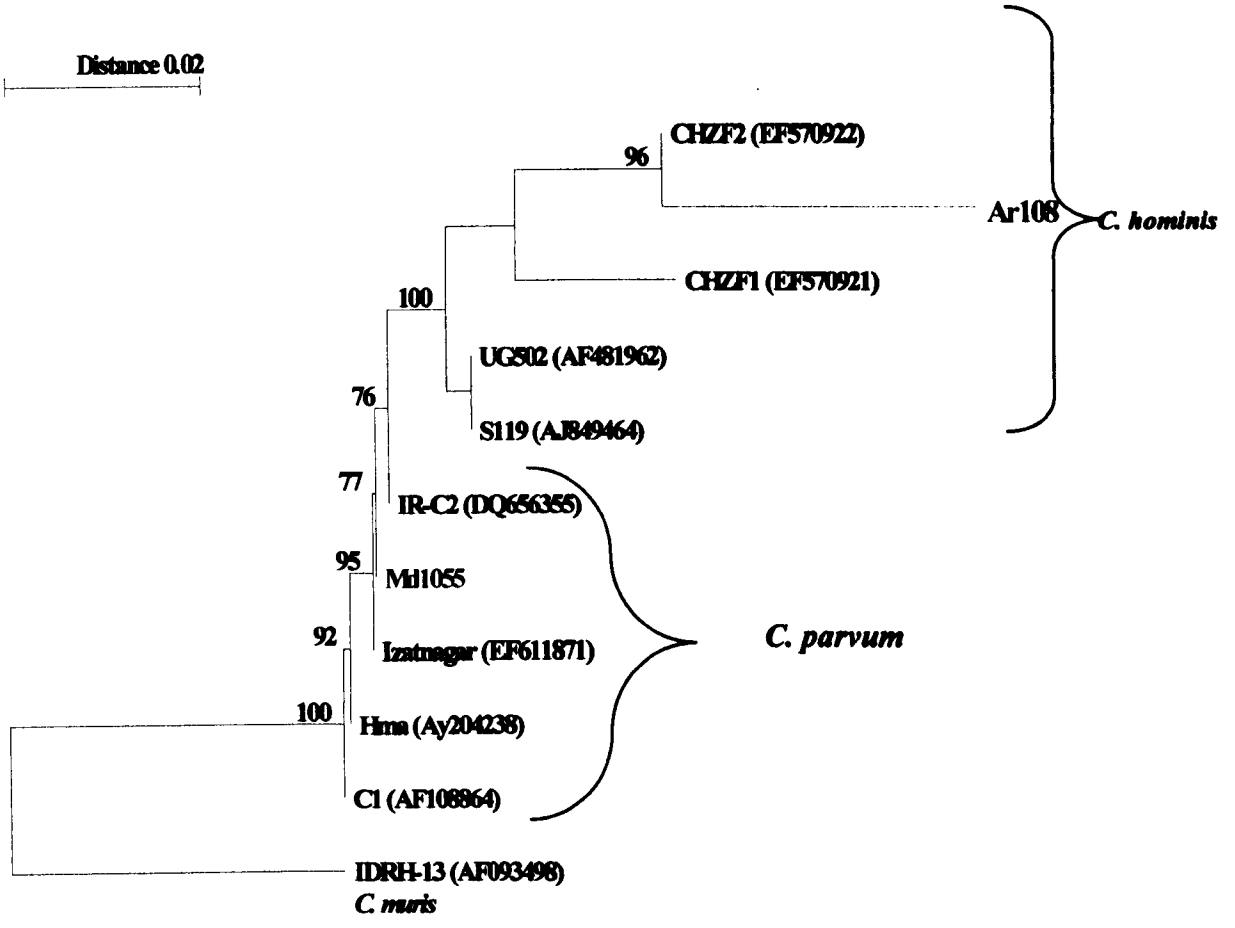


Figure 6.1. **Phylogeny of two *Cryptosporidium* isolates Ar108, which was identified as *C. hominis* and Md1055, which was identified as *C. parvum* and other reference strains by a rooted NJ-tree based on 18S rRNA gene. The numbers on branches are bootstrap values greater than 70%. The scale bar indicates an evolutionary distance of 0.02 nucleotides per position in the sequence. The reference sequences accession numbers are inserted between brackets.**

6.2.2.2. The phylogeny of the GP60 gene

C. hominis isolate 7505 Id was used from GenBank as the out-group as this species showed least similarity to the others (Figure 6.2). This species formed a separate clade with the full statistical reliability (100%) with another clade that consisted of *C. hominis* and *C. parvum* that are in different allele families. Our first sample Ar108 which was identified as *C. hominis* Ie is in one group with its reference strain from the GeneBank *C. hominis* Ie isolate 4302 (Accession number DQ665689) and they are supported as a group by a bootstrap value of 100%. The second sample Md1055 which was identified as *C. parvum* IId is in one group with its reference strain from the GeneBank *C. parvum* IId isolate 5635 (Accession number AY738186) but they are not statistically supported.

Both of the two samples Md1055 (*C. parvum*) and Ar108 (*C. hominis*) partial GP60 gene sequences have been deposited in GenBank under accession numbers AB369995 and AB369996 respectively.

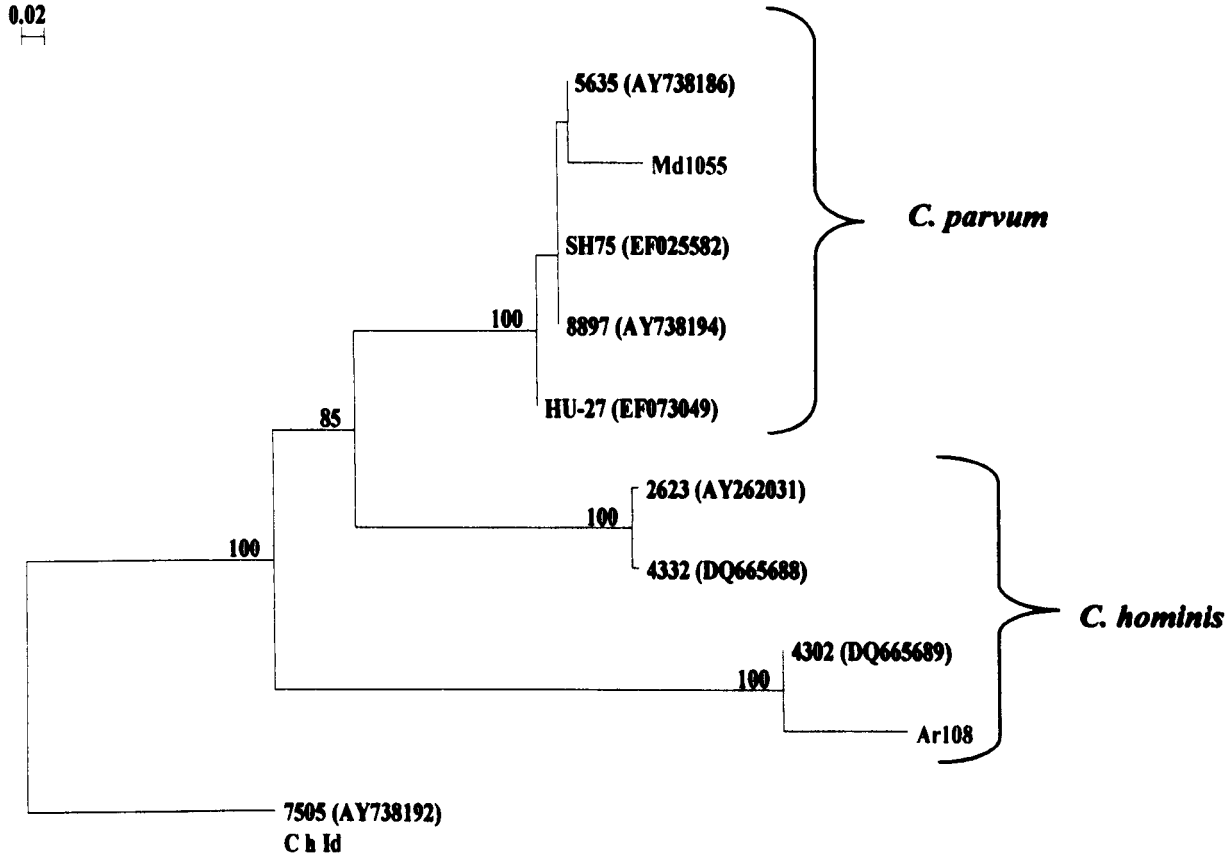


Figure 6.2. **Phylogeny of two *Cryptosporidium* isolates Ar108, which was identified as *C. hominis* and Md1055, which was identified as *C. parvum* and other reference strains by a rooted NJ-tree based on GP60 gene. The numbers on branches are bootstrap values greater than 70%. The scale bar indicates an evolutionary distance of 0.02 nucleotides per position in the sequence. The reference sequences accession numbers are inserted between brackets.**

6.2.2.3. The phylogeny of the HSP70 gene

C. meleagridis isolate 672 was used from the GenBank as the out-group as this species showed least similarity to the others (Figure 6.3). This species formed a separate clade with the full statistical reliability (100%) with another clade that consisted of *C. hominis* and *C. parvum*. Our first sample Ar108 which was identified as *C. hominis* is in one group with its reference strain from the GeneBank *C. hominis* isolate CHZF2 (Accession number EF591788) and they are not statistically supported as a group. The second sample Md1055 which was identified as *C. parvum* is in one group with its reference strain from the GeneBank *C. parvum* isolate IowaII (Accession number XM_625373) and they are supported as a group by a bootstrap value of 98%.

Both of the two samples Md1055 (*C. parvum*) and Ar108 (*C. hominis*) partial HSP70 gene sequences have been deposited in GenBank under accession numbers AB369997 and AB369998 respectively.

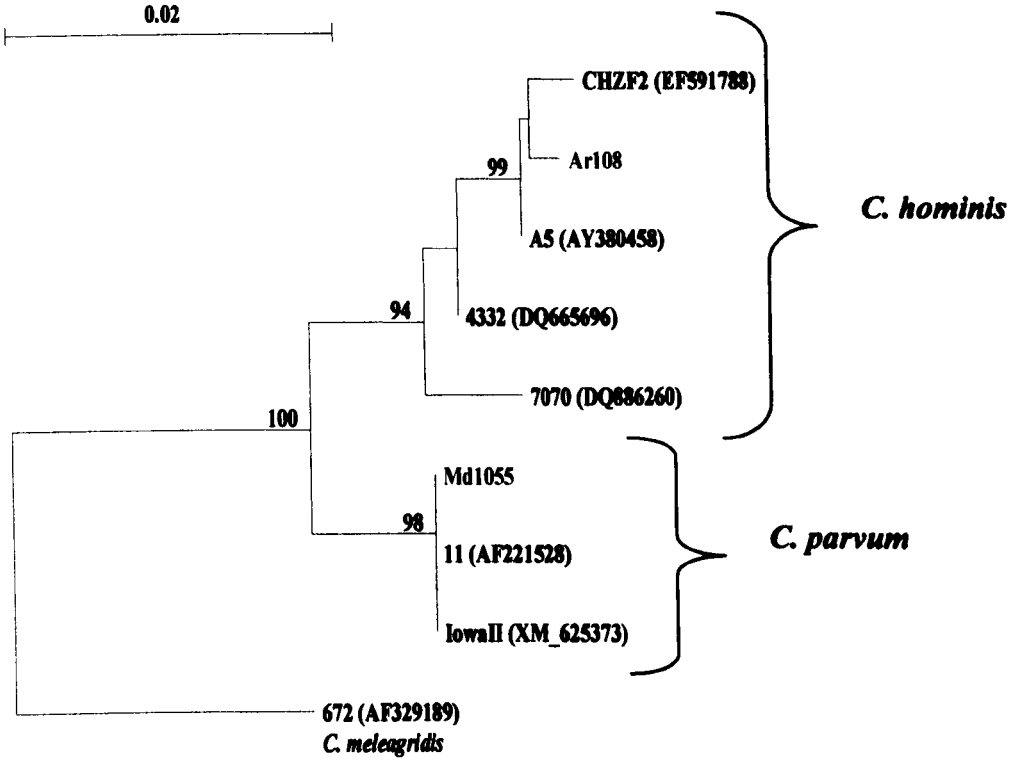


Figure 6.3. **Phylogeny of two *Cryptosporidium* isolates Ar108, which was identified as *C. hominis* and Md1055, which was identified as *C. parvum* and other reference strains by a rooted NJ-tree based on HSP70 gene. The numbers on branches are bootstrap values greater than 70%. The scale bar indicates an evolutionary distance of 0.02 nucleotides per position in the sequence. The reference sequences accession numbers are inserted between brackets.**

6.2.3. Cloning

Cloning of the 18S rRNA gene of the mixed infection samples was carried out as described in section 3.4.2 using the secondary PCR products for 18S rRNA gene. Each transformation experiment was successful when plated out and *E. coli* TG2 transformed with pGEM-T containing different PCR amplicons produced a mixture of blue and white colonies. The white colonies containing pGEM-T with the disrupted ß-galactosidase gene were picked.

The PCR amplifications for the 18S rRNA locus were examined from 8 colonies for each of the 8 mixed isolates (a total of 64 colonies). Extractions that yielded products of between 833-837 bp were digested by *Vsp*1 (Figure 6.4 shows an example of Vsp1 digestion). For each isolate some colonies exhibited *Vsp*1 restriction sites with fragments of 104 and 628 bp, which indicate the presence of *C. parvum,* while the others had fragments of 104 and 561 bp which is characteristic of *C. hominis*. Representative colonies for each species were selected for recovery of the DNA from transformed bacteria and sequence analysis.

Species identification of the mixed isolates based on the sequence analysis of the clones of the 18S rRNA gene coincide with the identification obtained using18S rRNA PCR-RFLP (Table 6.2). From all the mixed isolates, two different species could be identified except from isolates no. 3, 64 from Al-Ardah and 28 from Abu-Areesh where only one species could be identified due to failure to obtain sequence data for the other co-infecting strain.

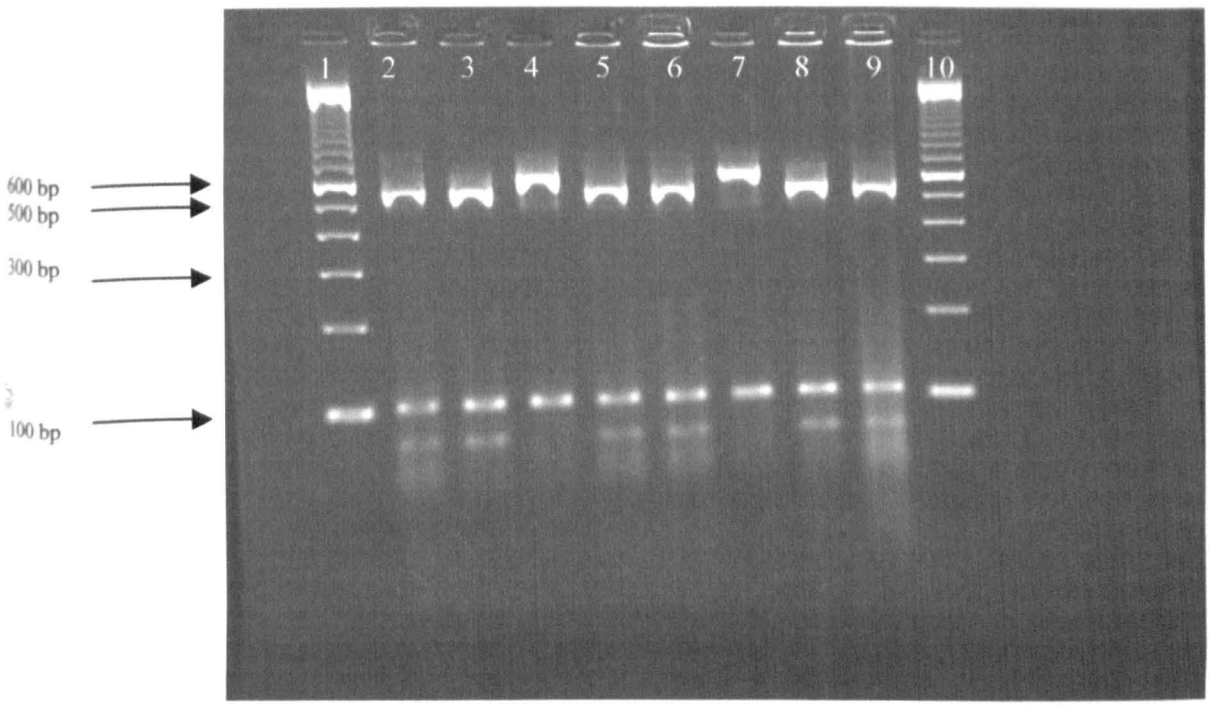


Figure 6.4. **Visualisation of RFLP of the 18S rRNA gene of isolate S2 colonies. Lanes 1&10 show the 1kb molecular weight markers. Lanes 4 & 7 showing the digestion with *Vsp*1 of 18S rRNA gene with bands for *C. parvum* and lanes 2-3, 5-6 and 8-9 with bands for *C. hominis*.**

Table 6.3. The results of species identification from the 8 mixed isolates based on the sequence analysis of the 18S rRNA clones with the remainder of the identification based on PCR-RFLP of the 18S rRNA gene

Sample no.	Source	Species identified based on PCR-RFLP of the 18S rRNA gene.	Species & genotypes identified based on sequence analysis of the 18S rRNA clones.
3	Al-Ardah	*C. parvum* + *C. hominis*	*C. hominis* CHZF2 (EF570922).
12	Al-Ardah	*C. parvum* + *C. hominis*	*C. parvum* HCTX8 (AF159111) + *C. hominis* CHZF2 (EF570922).
64	Al-Ardah	*C. parvum* + *C. hominis*	*C. parvum* HCTX8 (AF159111)
73	Al-Ardah	*C. parvum* + *C. hominis*	*C. parvum* HCTX8 (AF159111) + *C. hominis* CHZF2 (EF570922).
101	Al-Ardah	*C. parvum* + *C. hominis*	*C. parvum* CPRM1 (AF112569) + *C. hominis* NL2 (DQ388386).
130	Al-Ardah	*C. parvum* + *C. hominis*	*C. parvum* CPRM1 (AF112569) + *C. hominis* CHZF2 (EF570922).
2	Samtah	*C. parvum* + *C. hominis*	*C. parvum* IR-C2 (DQ656355) + *C. hominis* CHZF2 (EF570922).
28	Abu Areesh	*C. parvum* + *C. hominis*	*C. hominis* CHZF2 (EF570922).

6.3. 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 Gizan and Maddina, Saudi Arabia. It is also the first study that has investigated cryptosporidiosis among animals including sheep, goats and cows using molecular techniques. The results of this study are in agreement with other reports from UK (McLauchlin *et al.*, 2000) and France (Guyot *et al.*, 2001) that demonstrated a high prevalence of zoonotic species of *Cryptosporidium* including *C. parvum* in human infection.

During the mid to late 1980s it was discovered that the 18S rRNA gene sequence had several properties that render it useful in phylogenetic analysis(Olsen & Woese, 1993). The 18S rRNA genes are of an intermediate size 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* species, 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 and types of cryptosporidia present are unknown (Gatei *et al.*, 2003).

The present study has demonstrated a remarkable degree of genetic variation at the GP60 locus among *Cryptosporidium* isolates. These findings have confirmed the observations of several previous studies (Strong *et al.*, 2000; Sulaiman *et al.*, 2001; Leav *et al.*, 2002; O'Connor *et al.*, 2002; Sulaiman *et al.*, 2005). The present study confirms the presence of an isolate of *C. hominis* allele group Ib from a child in Maddina and this suggests that human to human or water to human transmission of cryptosporidiosis had taken place in Maddina children, Saudi Arabia. This allele group has been reported from

Milwaukee, USA (Zhou *et al.*, 2003a) and was the cause of six waterborne outbreaks and one food-borne outbreak in the USA and Northern Ireland as reviewed by Xiao *et al.*(2003). In this study, 6 children in Gizan were infected with an allele Ie subtype, indicating that anthroponotic transmission of this parasite occurs in Gizan. Nevertheless, the low proportion of infections due to *C. hominis* suggests that anthroponotic transmission of cryptosporidiosis in Saudi Arabia is probably not as important as in other countries.

Among *C. parvum* isolates of this study, allele group IId is the major group and has been found in Gizan and Maddina with high frequency. Interestingly, this allele group is unusual but it has been reported in six AIDS patients and three cattle in Portugal (Alves *et al.*, 2003) and in 29 children in Kuwait (Sulaiman *et al.*, 2005). Allele group IIc (formerly known as Ic) has been detected in two samples from Maddina and Gizan and has so far been isolated only from humans (Alves *et al.*, 2003). This allele group suggests human to human transmission of *C. parvum* (Chalmers *et al.*, 2005; Sulaiman *et al.*, 2005). Allele group IIa which has been identified in one isolate from Maddina has been reported in both humans and ruminants. In particular it has been reported in 28 children in Kuwait (Sulaiman *et al.*, 2005). Overall, 72 isolates of the 80 isolates detected by GP60 gene PCR could be of anthroponotic or zoonotic origin while the remaining 8 isolates were of anthroponotic origin.

Our study has also demonstrated the occurrence of mixed infections with *C. parvum* and *C. hominis* in 8 patients. Although humans are susceptible to both species, 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 12% *C. hominis* and *C. parvum* mixed infections among 135 human cryptosporidiosis cases from Scotland (Mallon *et al.*, 2003). Sequencing of

PCR clones from 3 isolates from Gizan failed to obtain sequencing data for the other co-infecting strain. A recent study in USA suggests that direct sequencing of multiple PCR products may be better than sequencing of PCR clones, as the latter can introduce sequence artefacts when mixed *Cryptosporidium* genotypes are present in samples (Zhou *et al.*, 2003b).

The *Cryptosporidium* HSP70 gene did not appear to be a good target for genotyping in spite of its high level of heterogeneity spread over the entire sequence that has been reported by an other study (Sulaiman *et al.*, 2000). In the present study, all the *C. parvum* isolates showed 98%-100% sequence identity to the published strain of Iowa II (Accession number XM_625373) while all the *C. hominis* isolates showed 98%-99% sequence identity to the published strain of CHZF2 (Accession number EF591788). Based on the phylogenetic trees of the three genes, 18S rRNA, GP60 and HSP70, the two representative samples Ar108 and Md1055 were identified as *C. hominis* and *C. parvum* respectively with higher bootstrap values with their reference strains for GP60 and HSp70 than 18S rRNA gene. This observation is supported by Sulaiman *et al.*(2000) who concluded that phylogenetic analysis of *Cryptosporidium* parasites based on HSP70 gene sequences is much more robust than 18S rRNA. One reason is that, unlike mutations in the 18S rRNA gene which occur in a restricted region of the gene, mutations in the HSP70 gene are spread over the entire sequence. In addition, the alignment of sequences is much easier because deletions and insertions are limited in the HSP70 gene.

In conclusion, the data generated in this chapter showed that the18S rRNA gene was useful for detecting the presence of *Cryptosporidium* spp. in unknown isolates, but had a low resolution of differentiation between isolates. While analysis of sequences from the GP60 locus revealed more subtypes in the sample set. This analysis suggests that this gene is more useful in the detailed differentiation of various isolates of *Cryptosporidium* spp.

7. GENERAL DISCUSSION AND CONCLUSIONS

Towards the end of the twentieth century, *Cryptosporidium* emerged as an important aetiologic agent of epidemic and endemic diarrhoeal disease worldwide, affecting mostly children and immunocompromized individuals (Fayer, 2004). Recognition of the existence of genetic diversity in *Cryptosporidium* has led to the development of molecular techniques for identification of morphologically indistinguishable species. This has helped researchers to understand the epidemiology and public health significance of *Cryptosporidium* spp. found in animals and the environment (Xiao *et al.*, 1999; Xiao *et al.*, 2004). Importantly, there have been very few reports of molecular studies of cryptosporidiosis in developing countries such as Saudi Arabia, where human infection with this intestinal parasite is a common health problem (Al-Braiken *et al.*, 2003).

In the first part of this study, microscopy identified *Cryptosporidium* oocysts in 5.7% of the 454 human stool samples collected from different areas in the Gizan region. SMB staining methods identified more *Cryptosporidium* oocysts compared to ZN, but the difference was not statistically significant (p=0.5) and the strength of agreement between the two methods was almost perfect (Kappa value=0.88). Similar results were obtained in the original report that compared SMB and ZN staining (Baxby *et al.*, 1984). These positive rates are as expected in Saudi Arabia, since two studies have reported prevalences using bright field microscopy of between 1% and 32% (Al-Braiken *et al.*, 2003, Khan *et al.*, 1988). The oocysts of *Cryptosporidium* were identified in only one sheep sample among 279 samples collected from domestic animals in the Gizan region. This very low detection rate could be due to using 10% formalin to preserve the samples so that they could be imported safely into the UK, and this may have reduced stainability and PCR detection of the oocysts. More likely, some of the samples were collected from non-diarrhoeal cases, but from animals with soft stools and this may add to the explanation for the low positivity rate. Alternatively, zoonotic activity of the parasite may be very low in this area, or the number of oocysts could be very low in the samples.

The infection prevalence rates observed in the present study would probably have been higher 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 be required (Navin & Juranek, 1984).

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 oocyst 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 SMB and ZN staining techniques, and this difference was statistically significant ($p=0.026$). However, there was a substantial degree of agreement between these tests when positive (Kappa value=0.71). 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 stool. However, the ELISA used in this study achieved the following: (i) infections with three species of the parasite (*C. hominis*, *C. parvum* and *C. felis*) were identified and (ii) identification of *Cryptosporidium* in one animal sample which had been preserved with 10% formalin.

Microscopic detection has the limitation that it is of little use in the 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). However, the method is useful as a baseline screening method when 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.

Based on our results, ELISA is the gold standard method since it was able to detect all the samples that have been detected by microscopy and PCR. Using the ELISA technique, the positivity rate of *Cryptosporidium* infection was nearly twice as frequent (9.91%) in Gizan compared to Maddina (5.2%), with no positive cases detected among the comparison group from the Gizan region. This might be due to differences in climate and the location of the two cities.

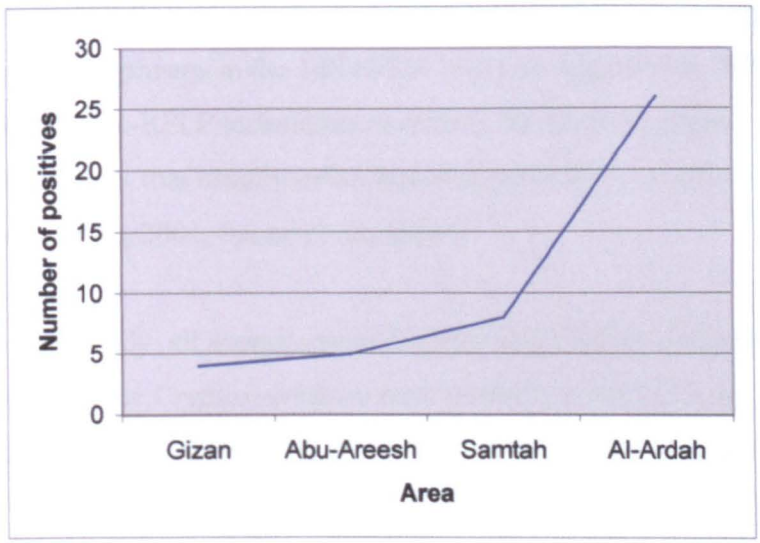
Interestingly, as the study areas within Gizan region become geographically closer to the capital city Gizan, the detection rate of *Cryptosporidium* infection became gradually lower (Figure 7.1). The detection rates were 20%, 8%, 8% and 4% in Al-Ardah, Samtah, Abu-Areesh and Gizan the capital city respectively. The main reason for this drop is probably the sanitary conditions in these areas, with the best being in the capital city, Gizan. Improvements in water, sanitation, household hygiene, socioeconomic standards and education might all have decreased the detection rate of the parasite in these areas.

The lowest *Cryptosporidium* detection rate was reported in Al-Khobah (4%), although this area is in close contact with the Yemen border from the south. This might be due to the fact that some samples were collected from older patients and the mean age of the cases in Al-Khobah was 151.7 months with a range between 5 to 960 months, compared to typical mean ages of 36 to 82 months in the other areas studied in Gizan region.

There was an obvious relationship between young age and cryptosporidiosis and the infection was more common in children less than two years of age in both cities. This

is in agreement with most epidemiological studies that 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).

Figure 7.1. The relationship between distance from Gizan City and the prevalence of cryptosporidiosis in patients with diarrhoea



Distance from Gizan City: Abu-Areesh (≈25 km), Samtah (≈33 km) and Al-Ardah (≈40 km)

The maximum prevalence of *Cryptosporidium* infection was during the cooler months in both Gizan and Maddina and the peaks of infection were in January and March respectively. In two studies from Kuwait, which has high temperatures during summer with a very dry climate and a short cool winter, similar to the climate in Saudi Arabia, the highest prevalence was during winter (March and April) (Daoud *et al.*, 1990) or from January to April (Iqbal *et al.*, 2001). A third study which was undertaken in Irbid, Jordan reported that the maximum prevalence was from January to May (Mahgoub *et al.*, 2004).

In the second part of this study, multilocus PCR-RFLP and sequencing were performed. To the best of our knowledge this is the first study to report on the identification of *Cryptosporidium* isolates in Gizan and Maddina, Saudi Arabia. Of 104 positive samples of *Cryptosporidium* spp. which were detected by light microscopy and/or ELISA screening methods, 101 (97.1%) samples yielded amplicons for the 18S rRNA gene. Failure to yield PCR products from the remaining three samples for the 18S rRNA gene could be related to variations in the primer binding region, which may occur in novel species and may result in lower specificity and amplification failures. Therefore, the methods may require further modification for specific species or genotypes because of the sequence polymorphisms in the 18S rRNA locus as suggested by Widmer *et al.* (1998). A variety of PCR-RFLP techniques have been developed to detect and distinguish the *Cryptosporidium* spp. that usually infect humans, particularly *C. parvum* and *C. hominis* (Sturbaum *et al.*, 2001, Spano *et al.*, 1998).

In the current study, all samples were initially amplified by 18S rRNA-based PCR. Positive samples for *Cryptosporidium* were further genotyped by an 18S rRNA-based PCR-RFLP technique (Xiao *et al.*, 1999). All samples were also amplified by PCR of GP60 (Zhou *et al.*, 2003) and HSP70 genes (Gatei *et al.*, 2006) for sub-genotyping. Some samples positive for 18S rRNA did not amplify using the GP60 and HSP70 primers. The presence of one copy of these genes per genome, in comparison with the presence of 5 copies of the 18S rRNA gene, and possible primer mismatches, are some possible explanations for the small failure rates of the PCR reactions for GP60 and HSP70 genes (Kato *et al.*, 2003, Alves *et al.*, 2003, Sulaiman *et al.*, 2001).

The 18S rRNA gene has been used extensively for the identification and characterisation of *Cryptosporidium* parasites (Peng *et al.*, 1997, Xiao *et al.*, 1999, Morgan *et al.*, 2000, 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 three different *Cryptosporidium* species from our study samples. By amplification and analysis of this gene fragment, we achieved positive

identification of *C. felis* from a sheep sample, in addition to detecting the more common *C. parvum* and *C. hominis* species. Our results have confirmed that the 18S rRNA gene target is an important tool for the precise identification of *Cryptosporidium* species, especially due to wide application of a single set of primers for the different species.

In the past, it has been assumed that, in areas where human *Cryptosporidium* infections are endemic, such infections are most likely to be caused by *C. hominis*, which predominates in most developed countries (Xiao *et al.*, 2000, Tiangtip & Jongwutiwes, 2002). However, our results indicate that *C. parvum* remains an important species infecting humans. This is in agreement with the published literature on human cryptosporidiosis (Morgan *et al.*, 2000, Xiao *et al.*, 2001, Tiangtip & Jongwutiwes, 2002). Our results showed that about 85% of the isolates from the study may be zoonotic in origin (*C. parvum*) and the rest could have been originally from humans. This finding is similar to results obtained in other studies elsewhere in the developing world (Morgan *et al.*, 2000, Alves *et al.*, 2001, Guyot *et al.*, 2001). Learmonth *et al* (2004) reported that a difference was observed between the *Cryptosporidium* species from rural and urban isolates, with *C. hominis* dominant in the urban region, whereas *C. parvum* was prevalent in rural New Zealand. Similarly, zoonotic *C. parvum* infections were more common in the rural northwest of the UK, compared to *C. hominis* predominating in the south of Britain (Hunter *et al.*, 2004). This may add to the explanation of the high detection rate of *C. parvum* in our study since the majority of the samples were collected from rural areas. Most PCR studies have led to the confirmation of *C. parvum* and *C. hominis* as the major causes of cryptosporidiosis in humans. However, recent reports have documented human infections with zoonotic species including *C. meleagridis* (Xiao *et al.*, 2001), *C. canis, C. felis* (Pedraza-Diaz *et al.*, 2001); *C. muris* (Gatei *et al.*, 2002), *Cryptosporidium* 'Cervine' genotype (Ong *et al.*, 2002) and *C. parvum* 'pig' genotype (Xiao *et al.*, 2002), which have been seen in both normal and immunocompromised hosts.

The high rates of *C. parvum* infections in humans suggests that farm animals, domestic pets and some wildlife can be potential sources, and this does not disagree with

our results because the low detection rate among the animal samples could be due to the reasons mentioned previously. Nevertheless, results of recent subtyping studies have shown the presence of human-adapted *C. parvum* subtypes, even in areas with intensive transmission of *C. parvum* between humans and farm animals (Alves *et al.*, 2003, Mallon *et al.*, 2003a, Mallon *et al.*, 2003b). Recently, Xiao and Feng (2008) mentioned that exclusive anthroponotic transmission of seemingly zoonotic *C. parvum* subtypes was seen in Mid-Eastern countries (Xiao & Feng, 2008). Our results confirm this, with the human adapted GP60 alleles (IIa and IIc) found in 3/74 (4.1%) of patients with *C. parvum* infections. Thus, not all *C. parvum* infections in humans are the result of zoonotic transmission (Xiao & Ryan, 2004).

Isolating *C. felis* from a sheep is one of the interesting results and this parasite can infect humans too. In a review by Raccurt (2007), 58 cases of human *C. felis* infection reported in different parts of the world were analysed. It was concluded that this emerging protozoan disease is present in humans around the world, with the exception of Australia and Oceania, and that both adults and children are infected, more often when immunocompromised by HIV infection (83% of reported cases). Apparently, immunocompetent individuals can also be infected by *C. felis*. In developing countries, inhabitants are more likely infected by *C. felis,* probably through the oocyst contamination of drinking or recreational water. The public health importance of *C. felis* infection in tropical countries remains to be evaluated (Raccurt, 2007).

Mixed *Cryptosporidium* species infections were found in 8 patients (7.9%) using 18S rRNA-RFLP. Interestingly, most studies have reported none or only an extremely low number of cases where both species were found together in the same host (Carraway *et al.*, 1997, McLauchlin *et al.*, 1999, Patel *et al.*, 1998, Quiroz *et al.*, 2000, Tumwine *et al.*, 2003) (Table7.1). Tumwine *et al.* (2003) found that among 444 children infected with *Cryptosporidium* spp., 19 (4.3%) were infected with a mixture of *C. hominis* and *C. parvum,* while we found 8/101 (7.9%)

Table 7.1. Comparing the proportions of mixed infections with *C. parvum* and *C. hominis* in this and other studies

Detection rate	Reference
2/15 (13.3%)	(Carraway *et al.*, 1997)
0/218 (0%)	(McLauchlin *et al.*, 1999)
2/46 (4.3%)	(Patel *et al.*, 1998)
0/59 (0%)	(Quiroz *et al.*, 2000)
18/444 (4.1%)	(Tumwine *et al.*, 2003)
8/101 (7.9%)	**Our result**

C. parvum isolates from both Gizan and Maddina, Saudi Arabia showed some variation on the basis of GP60 gene sequence. The presence of *C. parvum* and *C. hominis* indicates both human and animal transmission cycles of cryptosporidiosis in Saudi Arabia. Results of subtype analysis further support the complexity of human pathogenic *Cryptosporidium*, and five subtype alleles of *C. parvum* and *C. hominis* were found in the present study. Subtype alleles Ie and Ib are characteristic of *C. hominis* and thus were likely of human origin, whereas IIa and IId are linked to *C. parvum* which could have originated from domestic and wild ruminants or humans. However, allele IIc which is linked also to *C. parvum*, formerly known as Ic, has so far been detected only in humans (Alves *et al.*, 2003).

The results of sequence analysis suggest that multiple routes of transmission of cryptosporidiosis in Saudi Arabia were probably responsible for the high genetic heterogeneity of *Cryptosporidium* parasites. For instance, *C. hominis* allele group Ib may indicate human to human or human to water transmission (Zhou *et al.*, 2003), and allele group IIc may indicate the human to human transmission of *C. parvum* (Sulaiman *et al.*, 2005, Chalmers *et al.*, 2005). In our study, the evolutionary distances in *C. hominis* isolates were very high and this is in agreement to Alves and Colleagues (2003) whom

reported that *C. parvum* parasites from humans (C. *hominis*) had an even higher genetic diversity than those from animals. The expansion of *C. parvum* into humans may be due, in part, to the historic development of intensive husbandry practices for various livestock species and the associated high concentrations of young animals at feeding operations. The clinical outcome of *C. parvum* and *C. hominis* infections in experimentally infected antibiotic pigs appears to be determined in part by the *Cryptosporidium* spp. used. *C. parvum* strains have shorter prepatent periods (3-6 vs 8-12 days) and have elicited more disease than *C. hominis* strains in this model and there is evidence for different clinical patterns of disease in humans (Pereira *et al.*, 2002).

Based on our results, sequencing of the Gp60 gene PCR products is the most discriminatory test for research studies, because it showed a remarkable degree of genetic variation among *Cryptosporidium* isolates from both Gizan and Maddina, Saudi Arabia. In comparison, sequencing of the HSP70 gene PCR products did not reveal any genetic variation among *Cryptosporidium* isolates from either geographical region. For clinical approaches, ELISA with one of the staining methods, SMB or ZN is enough to diagnose the parasite.

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*. Although nitazoxanide and azithromycin have shown some promise as effective drugs (Doumbo *et al.*, 1997; Smith, 1998; (Rossignol *et al.*, 2006), there are no fully effective therapeutic agents. Control and treatment depend upon early and accurate diagnosis and a full understanding of the epidemiology and transmission dynamics, highlighting the need for preventive measures. The accurate identification of *Cryptosporidium* to species, genotype and sub-genotype levels 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 for 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 persistence of oocysts 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).

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.

Conclusions and recommendations for future studies:

Future studies should focus on zoonosis, particularly in the Gizan region, where there are large numbers of animals. Animal samples should be collected and directly examined the same day, without using any chemical preservative, to avoid oocyst degradation. This may add to the explanation of the large number of *C. parvum* detected in both Gizan and Maddina.

Human studies should focus predominantly on those in the age group <7 years, as age-related prevalences in this study are consistent with other studies in the Middle East region and elsewhere.

The period of the study should be at least 12 months, in order to be able to detect any seasonal variation.

For the detection of *Cryptosporidium* spp., ELISA, microscopy with ZN or SMB and 18S rRNA gene PCR followed by RFLP can be used for screening. GP60 gene PCR can be used for the subtyping of the isolates to provide valuable information about all circulating *Cryptosporidium* species and strains in the region.

Better education and increased awareness of cryptosporidiosis among the general public could potentially reduce case numbers (Robertson *et al.*, 2002).

Finally, we recommend that physicians should be made aware of *Cryptosporidium* as a cause of diarrhoea in children, and that adequate procedures for the detection of this parasite should be included in the routine diagnostic processing of diarrhoeal stool specimens in all clinical laboratories. This is particularly important as more effective drugs such as nitazoxanide (Rossignol *et al.*, 2006) become available for therapy.

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APPENDIXES
1-A, 1-B & 1C

Kingdom OF Saudi Arabia
Ministry Of Health
HEALTH, AFFAIRS, GAZAN
HOSPITAL ADMINISTRATION, DEPTT

المملكة العربية السعودية
وزارة الصحة
المديرية العامة للشؤون الصحية بمنطقة جازان
إدارة المستشفيات

الرقم : ٨٦٠ /٤/٥١ التاريخ : ٦ / ٨ /١٤٢٥هـ المشفوعات : ٢

سعادة مدير مستشفى الملك فهد ا لمركزي / جازان العام/ابو عريش العام/ صامطه العام/ العارضه العام/ مركز الرعاية الاوليه بالخوبه المحترمين

السلام عليكم ورحمة الله وبركاته

إشارة إلى الطلب المقدم من الباحث/محمد يحي عريشي والمبني على إفادة الملحق الثقافي السعودي البريطاني بخصوص الدراسة الحقلية المتعلقة بدرجة الدكتوراه في الأحياء الدقيقة الطبية من كلية الطب بجامعة ليفربول في بريطانيا في مجال التشخيص الجزيئي للطفيليات المسببة للإسهال في الأطفال والمضعفين مناعيا

عليه نأمل التعاون مع الباحث وتسهيل مهمته.

وتقبلوا أطيب تحياتي.

مساعد المدير العام لشئون المستشفيات

د. محسن بن عبده عداوي

ص/للصادر

م/عريش

FAX 073170416 فاكس TEL 073221418 تلفون

وزارة الصحة
المديرية العامة للشئون الصحية بالمدينة المنورة
إدارة التخطيط والتطوير / شعبة التدريب والابتعاث
46/16

التاريخ : ١٤٢٥/٦/٩
المرفقات :
الموضوع :

سعادة مدير عام مستشفى الملك فهد بالمدينة المنورة الموقر
السلام عليكم ورحمة الله وبركاته .

نرفق لكم بطيه صورة من قرار مدير عام شئون الموظفين بالوزارة رقم ٢٩/١/٨٤٤٣٨ وتاريخ ١٤٢٥/٥/٢٣هـ والمتضمن الموافقة على قيام أخصائي المختبر علي محمد علي خيمي برحلة علمية إلى المملكة العربية السعودية لمدة ثلاثة أشهر اعتباراً من ١٤٢٥/٩/١٩هـ وحتى ١٤٢٥/١٢/١٨هـ ليتمكن من إكمال بحثه للحصول على درجة الدكتوراة

عليه نامل الإطلاع وتمكين المذكور من ذلك ..

وتقبلوا أطيب تحياتي ،،،

مدير ادارة
التخطيط والتطوير بصحة المدينة المنورة

عبدالله العتيبي

سهل بن عبد الوهاب سنبل

عادل العطي ١٤٢٥/٦/٤هـ

اعتماد للتعميم لمدير المختبر
اعتماد للتعميم لشعبة الاستحقاقات
١٧/٦
ملف المذكور
١٧/٦

بسم الله الرحمن الرحيم

Royal Embassy of Saudi Arabia
Cultural Bureau
London/UK

المملكة العربية السعودية
وزارة التعليم العالي
مكتب الملحق الثقافي في بريطانيا

الأكاديمية

رقم الملف: A 275

الأخ المبتعث الكريم / محمد يحي عريشي **المحترم**

السلام عليكم ورحمة الله تعالى وبركاته، وبعد

إشارة إلى طلبك الموافقة على القيام برحلة علمية لمدة ثلاثة أشهر للمملكة وذلك لجمع المادة العلمية المتعلقة بدراستك.

نفيدك بورد خطاب سعادة وكيل وزارة التعليم للعلاقات الثقافية المكلف رقم ب/٤/١٤٤/١٨٩٥٤ وتاريخ ١٤٢٥/٧/١٦هـ والمتضمن الموافقة على قيامك بالرحلة العلمية المشار إليها.

كما نود إحاطتك بضرورة تزويدنا بجواز سفرك وتقرير من الجهة التي تولت الإشراف عليك أثناء الرحلة العلمية يوضح تاريخ بداية ونهاية الرحلة حال عودتك إن شاء الله وذلك حتى نتمكن من صرف المخصصات المالية المتعلقة بها.

نأمل الاتصال بالمكتب لتكملة إجراءات سفرك.

مع أمنياتنا لك بالتوفيق،،،

الملحق الثقافي في بريطانيا

عبدالله بن محمد الناصر

ن م

NO. ٤/٤٢٥٧ DATE: ٢٢/٧/٤٢٥ ENC. ---

29 BELGRAVE SQUARE LONDON SW1X 8QB - Telephone: 0207-245 9944/5/6 - Fax: 0207-245 9895

Appendix 1-D
نموذج استبيان ومعلومات المرضى

إسم المستشفى:

مسلسل	رقم ملف المريض	العمر	الجنس	تاريخ أخذ العينة	التشخيص

Appendix 1-E

ورقة معلومات الوالدين لأطفال المدارس

الموضوع: كريبتوسبوريديوسيز في منطقة جيزان-المملكة العربية السعودية

يحدث هذا المرض نتيجة الإصابة بطفيل صغير يسمى كريبتوسبوريديوم يتسبب في اسهال وأحيانا حمى وانخفاض الوزن في الأطفال المصابين لأن الطفيل يتواجد في أحشاء الإنسان وكذلك الحيوان. ويكون المرض أشد ضراوة في الأطفال قليلي المناعة حيث تستمر اصابتهم فترة أطول وفي حالات نادرة قد تؤدي إلى الوفاة.

ينتقل هذا الطفيل عن طريق البراز في حالة تلوث مياه الشرب و الطعام به ولكون الطفيل مقاوم جدا للكلور فإن هذا يساعده على العيش في الماء لينتشر في المجتمعات.

يسبب الإسهال الناتج عن الإصابة الجفاف وكذلك حالات فقد الوزن في الأطفال ولا يوجد علاج محدد حاليا للطفيل ولكن هناك بعض الأدوية التي قد تساعد كثيرا في اضعاف الطفيل و تخفيف الأعراض .

يعتمد التشخيص على الإختبارات المجهرية للبراز والتي يتم فيها البحث عن الطور المعدي للطفيل.

الهدف من هذه الدراسة هوالبحث عن وجود العدوى بالطفيل في منطقة جازان ومعرفة فصيلته ان وجد.

في حالة موافقتكم على اشتراك طفلكم بهذه الدراسة نأمل جمع عينة براز منه ووضعها في الوعاء البلاستيكي المرفق باستخدام القفازات المرفقة أيضا.

لكم كل الحق في رفض المشاركة وهذا لن يؤثر على العناية بطفلكم في أي حال.

PUBLICATIONS

review

Cryptosporidiosis in Saudi Arabia and neighboring countries

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Cryptosporidium is a coccidian protozoan parasite of the intestinal tract that causes severe and sometimes fatal watery diarrhea in immunocompromised patients, and self-limiting but prolonged diarrheal disease in immunocompetent individuals. It exists naturally in animals and can be zoonotic. Although cryptosporidiosis is a significant cause of diarrheal diseases in both developing and developed countries, it is more prevalent in developing countries and in tropical environments. We examined the epidemiology and disease burden of *Cryptosporidium* in Saudi Arabia and neighboring countries by reviewing 23 published studies of *Cryptosporidium* and the etiology of diarrhea between 1986 and 2006. The prevalence of *Cryptosporidium* infection in humans ranged from 1% to 37% with a median of 4%, while in animals it was different for different species of animals and geographic locations of the studies. Most cases of cryptosporidiosis occurred among children less than 7 years of age, and particularly in the first two years of life. The seasonality of *Cryptosporidium* varied depending on the geographic locations of the studies, but it was generally most prevalent in the rainy season. The most commonly identified species was *Cryptosporidium parvum* while *C. hominis* was detected in only one study from Kuwait. The cumulative experience from Saudi Arabia and four neighboring countries (Kuwait, Oman, Jordan and Iraq) suggest that *Cryptosporidium* is an important cause of diarrhea in humans and cattle. However, the findings of this review also demonstrate the limitations of the available data regarding *Cryptosporidium* species and strains in circulation in these countries.

Cryptosporidium is a coccidian protozoan parasite of the intestinal tract that causes severe potentially fatal watery diarrhea in immunocompromised patients and self-limiting but often prolonged diarrheal disease in immunocompetent individuals.[1,2] It can also infect other animal species and thus may be zoonotic.[3,4] The infective form of *Cryptosporidium* is the thick-walled oocyst which is excreted in large numbers in the feces during acute infection. The oocyst is resistant to desiccation and most disinfectants.[5] Volunteer studies have demonstrated that the infective dose can be as low as one oocyst[5] and thus infection is easily spread person-to-person directly or indirectly.

Cryptosporidiosis is a significant cause of diarrheal diseases in both developing and industrialized nations,[6] but several epidemiologic studies have demonstrated that *Cryptosporidium* is more prevalent in developing countries (5% to >10%) than in developed countries (<1% to 3%).[7-9]

In temperate climates cryptosporidiosis accounts for only 1% to 4% of the cases of childhood diarrhea and is usually self-limiting.[5] It can, however, produce devastating diarrhea in HIV-infected individuals who have fewer than 200 CD 4+ lymphocytes/μL of blood.[10] In tropical environments however, cryptosporidiosis is much more prevalent accounting for 4% to 20% of the cases of childhood diarrhea, and children less than two years old have the greatest prevalence of cryptosporidiosis.[5,11] It is associated with high morbidity[8,12-14] and this probably reflects a poorer nutritional status in children in developing countries.[15] Transmission of *Cryptosporidium* spp via contaminated drinking water, outdoor and indoor recreational waters and municipal water is well documented.[16,17] Recently there has been

a large water-borne outbreak involving over 800 inhabitants of Bani Hassan in Jordan apparently associated with contaminated drinking water.

Recent molecular epidemiologic studies of cryptosporidiosis have helped us to gain better understanding of the transmission of cryptosporidiosis in humans and the public health significance of *Cryptosporidium* spp. in animals and the environment.

Using genotyping tools, five species of *Cryptosporidium* (*C. hominis, C. parvum, C. meleagridis, C. felis, and C. canis*) have been shown to be responsible for most human infections.[6] The potential for zoonotic infection is a genuine problem since *Cryptosporidium* lacks species specificity.[18] Therefore, domestic and wild species of mammals and birds may be reservoirs of infection for susceptible human individuals, whether they are immunodeficient or immunologically competent.[19] Diarrheal disease is a frequent illness in developing countries, and contributes to the death of 4.6 to 6 million children annually in Asia, Africa and America.[20] Cryptosporidiosis has been recorded as a cause of diarrhea in veterinarians and animal handlers,[21] in bone-marrow transplant recipients,[22] in household contacts of infected patients,[4,23] in homosexual men,[24] in travellers,[25] in children in day care centers[26-29] and in patients with chronic diseases.[30]

Methods for detection of Cryptosporidium spp in feces usually involve microscopic examination of stained fecal smears (modified Ziehl-Neelsen, safranin methylene blue, auramine-phenol), antigen detection (immunofluorescence, ELISA) or genome detection (PCR amplification of the 18S rRNA gene).[5] Each varies in sensitivity and specificity and there is no universally accepted "gold-standard".

There are few published reports of studies on cryptosporidiosis in the Middle East. In Saudi Arabia, diarrheal disease is an important cause of morbidity in children but the contribution made to it by *Cryptosporidium* spp is largely unknown.

The aims of the present review were:

(1) To document the prevalence of *Cryptosporidium* spp in humans and animals in Saudi Arabia and neighboring countries.
(2) To estimate the disease burden.
(3) To examine the age distribution of the cases.
(4) To determine the seasonality of cryptosporidiosis.
(5) To compare the importance of *Cryptosporidium* spp as a cause of diarrhea relative to other enteropathogens.

METHODS

Articles were identified for this review from a multilingual *MEDLINE* search for publications from 1976 to 2006 using the keywords *Cryptosporidium*, and the name of each of the following countries: Saudi Arabia, Kuwait, Iraq, Jordan, Bahrain, Qatar, Yemen, Oman and United Arab Emirates. In addition, the *Annals of Saudi Medicine* and the *Saudi Medical Journal* were handsearched for papers on diarrheal diseases and *Cryptosporidium*.

The studies were grouped into three categories: studies targeting human populations, studies targeting animal populations and studies identifying *Cryptosporidium* species and typing and these were analyzed separately. For each study, the prevalence of *Cryptosporidium* infection was examined initially in patients and compared with controls where a control group was included. Age profile and seasonality were also examined where sufficient data were available. Finally, we reviewed studies in which molecular characterization, typed species and subtyping allele families were available to examine the distribution of the different *Cryptosporidium* species and strains in circulation.

RESULTS

Description of Studies

We found 23 papers published from the region between 1986 and 2006. The prevalence of *Cryptosporidium* infection among humans was addressed by 15 papers,[10,31-44] while the prevalence among animals was addressed by two papers.[42,45] In addition, 5 papers identified the circulating species and reported the molecular characterization of the parasite.[6,36,46-48] One paper reported an outbreak of caprine cryptosporidiosis in the Sultanate of Oman and another examined the prophylactic value of paromomycin in the outbreak.[49,50] Finally, one paper described a case of cryptosporidiosis in a child in Kuwait.[51] The studies included five from Saudi Arabia[10,31,32,45,46] and the remainder from four neighboring countries: Kuwait,[6,33-35,51] Jordan,[36-40,44,47] Iraq[41-43,48] and The Sultanate of Oman.[49,50] Ten of these studies were hospital-based[6,31,33,34,36,40,41,43,48,51] four studies were designed to select inpatient cases only,[33,40,41,51] whereas both inpatients and outpatients were included in another four studies[31,34,36,43] and seven studies targeted hospital outpatients only.[10,32,35,39,44,46,48] In addition seven studies were entirely community-based (Table 1).[37,38,42,45,47, 49,50]

Study duration was variable, ranging from 3 months[10,34] to 38 months.[6] The median study duration was 12 months. The ages of the patients surveyed varied significantly, ranging from newborn (0-1 month)[31] to 87 years[38] and even within the same study the age groups ranged from 1 to 87 years.[38] Animal ages were reported

only in the study from The Sultanate of Oman[49] and they ranged between two days to over one year.

Detection methods

In most studies fecal specimens were screened by examining stained fecal smears by bright field microscopy and the most common stains used were acid fast stains, (16 studies)[10,32,36-39,41-50] followed by trichrome, (7 studies)[33,34,37-40,44] and safranin methylene blue, (5 studies).[31,33-35,51] Fluorescent microscopy was used to examine fecal smears stained with auramine-phenol, (4 studies).[32,40,49,50] Other techniques such as a direct immunofluorescence assay (DFA) were used in three studies[35,36,47] while polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) were used in one study from Kuwait.[6] Different techniques used prior to the screening were sedimentation, sugar flotation concentration and direct wet mount preparations.

Prevalence and Seasonality

In humans the prevalence of cryptosporidiosis ranged from 1.0%[32] to 37.3%[36] and seemed to be higher in the studies that targeted infants and children under 7 years old (Table 1) while in animals it was different for different species of animals and the geographic locations of the studies (Table 2). The prevalence of cryptosporidiosis differed depending on the population surveyed. Outpatient studies showed significantly higher prevalences than those where inpatients were included. In addition, mixed inpatient and outpatient studies showed higher prevalences than the community-based studies. The range and median of the outpatient prevalence studies were 1 to 32% and 6.7% respectively, whereas the range and median of combined inpatient and outpatient studies were 1.15 to 37.3% and 5.5% respectively.

Cryptosporidium infection was more common in children less than 7 years of age. Data from eight studies showed that the prevalence of cryptosporidiosis was higher in children less than 7 years old,[10,31,32,35,36,40-42,44] and 4 of these studies showed that the highest prevalence was among children less than 2 years of age.[10,32,35,40]

The seasonal variation in cryptosporidiosis was reported in five studies only. Two studies from Kuwait, which has high temperatures during the summer with a very dry climate and a short cool winter, showed that the highest prevalence was during winter (March and April)[33] or from January to April.[35] The other three studies were from Jordan, which has a relatively wet rainy season from November to April and very dry weather for the rest of the year with uniformly hot dry summers and cool variable winters. The maximum prevalence in the first study, which was done in Irbid City, was undertaken from January to May in the rainy season,[36] while in the two remaining studies, which were done in Badia, the maximum prevalence was reported in the warm months from May to September.[38,39]

Cryptosporidium species and strains identified

The most commonly identified species was described as *Cryptosporidium parvum*, which has been reported from Dammam, Saudi Arabia,[46] Kuwait,[6] Irbid, Jordan,[36] Basra, Iraq[48] and Bani-Kenanah in Jordan (Table 3).[47] *Cryptosporidium hominis* was reported in one study from Kuwait only.[6] Light and immunofluorescent microscopy were used to identify *Cryptosporidium* species in these studies except in the study from Kuwait where advanced molecular tools were used, namely PCR, RFLP and DNA sequencing. It is not possible to assign cryptosporidia to species on the basis of microscopic morphology alone.

In the last study,[6] four subtype allele families (IIa, IId, IIc and IIf) of *Cryptosporidium parvum* were identified and 3 (Id, Ie and Ib) of *Cryptosporidium hominis*. An atypical outbreak of caprine cryptosporidiosis occurred in the Sultanate of Oman in goats ranging in age from two days to adulthood, on a well-managed closed farm.[49] None of the other animals on the farm, including sheep, cows and buffalo were affected. Morbidity approached 100 per cent in goats less than six months of age. Despite intensive supportive care, 238 goats, ranging in age from two days to over one year, died. Cryptosporidia were detected in large numbers in the intestinal contents of dead animals and in faecal smears of animals with diarrhea.

In another study from the Sultanate of Oman, the prophylactic value of paromomycin (an aminoglycoside antibiotic analogous in structure to neomycin)[52] was examined in the same outbreak of caprine cryptosporidiosis.[50]

DISCUSSION

In the 23 studies addressing *Cryptosporidium* infections in Saudi Arabia and neighboring countries, *Cryptosporidium* infection in humans ranged between 1%[32] and 37.3%,[36] while in animals it was different for different species of animals and the geographic locations of studies. It appears that the prevalence of cryptosporidiosis in humans is lower than in animals.[42] Interestingly, the prevalence differed even within Irbid, a city in Jordan, where the prevalence varied from 1.5%[40] to 37.3%.[36] This may be due to methodological differences, as bright field microscopy only was used in most of the studies with its low detection rate compared

Table 1. Cryptosporidium detection rates in Saudi Arabia and neighboring countries.

Country	Period	Detection methods	Number of patients	Age of patients	Number of control	Age	Setting	Prevalence in patients (%)	Prevalence in control (%)	Seasonality	Reference
Saudi Arabia	3/00-5/00	S, DWM, BFM (AFS)	63	17% <1 y	190	<5 y	(O)	32	4.7	NR	(10)
Saudi Arabia	1990	S, BFM (SM-B)	174	25-59 mo	50	0-120 mo	H (I,O)	1.15	0	NR	(31)
Saudi Arabia	11/86-5/87	DWM, S, BFM (AFS), FM (AP)	209 * 112 **	2 y	0	0-12 y >12 y	(O)	1 0	0	NR	(32)
Kuwait	1/88-6/99	DWM, S, BFM (SM-B), TSSP	2205	5-96 mo	0	2 wk to 12 y	H (I)	1.6	0	Winter Mar, Apr	(33)
Kuwait	mid-Jan to mid-Apr 89	S, DWM, BFM (SM-B), TSSP	738 * 413 **	1.25-8 y	0	NR	H (I,O)	1.36 0	0	NR	(34)
Kuwait	9/95-8/97	S, BFM (SM-B), DFA	3549	>2 y	500	3 mo to 13 y	(O)	1.43	0	Winter Jan to Apr	(35)
Jordan	11/00-9/01	DWM, SFC, BFM (AFS), DFA	300	5-7 y	0	0 to 12 y	H (I,O)	37.3	0	Jan to May, the rainy season	(36)
Jordan	9/92-3/93	DWM, S, SFC, BFM (AFS, TSSP)	1000	<9 y	0	6 to 14 y	CB	4	0	NR	(37)
Jordan	9/99-9/01	DWM, S, SFC, BFM (AFS,TSSP)	200	mean 7.5 y	0	1 to 14 y 15 to 87 y	CB	5 3	0	Spring	(38)
Jordan	NR	DWM, S, BFM (AFS, TSSP)	180	NR	100	12 – 84 y	(O)	8.3	0	Warm mo May-Sep	(39)
Jordan	5-8/93	S, BFM (TSSP), FM (AP)	265	<1 y	0	< 5y	H (I)	1.5	0	NR	(40)
Iraq	11/97-5/98	S, BFM (AFS)	40	46- 55 y	175	2 y - 60 y	H (I)	5	1.14	NR	(41)
Iraq	NR	S, BFM (AFS)	60	<6 y	175	4-62 y	CB	5	1.14	NR	(42)
Iraq	1-12/00	S, BFM (AFS)	205	26-35 y	175	2 mo- 65 y	H (I,O)	9.7	1.1	NR	(43)
Jordan	7/92-9/93	DWM, SFC, BFM (TSSP, AFS)	300	<3 y	300	6 mo-6 y	(O)	6.7	1.7	NR	(44)

S: sedimentation, DWM: direct wet mount, BFM: bright field microscope, FM: fluorescent microscope, DFA: direct immunofluorescent assay, SFC: sugar flotation concentration, H: hospital-based, I: inpatient, O: outpatient, CB: community-based, * children, ** adults, SM-B: safranin methylene blue, AP: auramin-phenol, TSSP: trichrom stool smear preparation, AFS: acid fast stain (modified Ziehl-Neelsen, modified Kinyoun's), NR: not reported.

to DFA which is more sensitive. For example, in Irbid the detection rate was 37.3% in the study where DFA was used together with bright field microscopy,[36] while in an other study in the same city where bright field microscopy alone was used, the detection rate decreased to 1.5%.[40]

The finding that most *Cryptosporidium* infections occur among children less than 7 years of age is consistent in most studies in the region and is comparable with the reports from other parts of the world.[53-55] It is possible that the infection rate in these studies would have been higher if more than one stool specimen had been collected from each child because of the intermittent nature of oocyst excretion with this parasite.[56,57] Outpatient studies showed significantly higher prevalences than those where inpatients were included, which may have resulted from the presence of *Cryptosporidium* oocysts in asymptomatic children, some of whom could be considered carriers who act as important reservoirs of the organism and finally a potential source of infection.

Seasonal variations in prevalence have been noted in some studies.[33] Several factors could account for seasonal variations in the occurrence of cryptosporidiosis, including factors affecting the numbers 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 centers. However, in most studies, the highest numbers of cases were detected during the rainy season.[58,59] In Kuwait for example the climate is characterized by long dry hot summers (40-50 °C) and short warm winters. There is no rainy season in Kuwait as such. The highest prevalence was during winter (March and April)[33] or from January to April.[35] While in Jordan, which has a relatively moist rainy season from November to April and very dry weather for the rest of the year with hot dry uniform summers and cool variable winters, the maximum prevalence was from January to May. The highest prevalence was during the rainy season in Irbid[36] but in the warm months from May to September in Badia.[38,39]

Few studies have tried to identify the infecting species and *Cryptosporidium parvum* was the most prevalent species identified, but this was with one exception by microscopy alone. *Cryptosporidium hominis* was identified in a study from Kuwait[6] where RFLP was used. Because the majority of these studies were done between 1986 to 1998 no advanced molecular tools were used to identify the infecting species and the researchers depended completely upon microscopy and direct im-

Table 2. Cryptosporidium detection rates among animals in Saudi Arabia and neighboring countries.

Country	City	Period	Duration	Animal type	Detection methods	Number of animals	Prevalence	Reference number
Saudi Arabia	Al-Ahsa region	Oct 2002 to Oct 2003	13 mo	White-cheeked bulbuls	BFM (AFS)	42	28.6 %	(45)
Iraq	Basrah	NR	NR	Domestic animals	S, BFM (AFS)	198	13.6 %	(42)

BFM: bright field microscope, AFS: acid fast stain (modified Ziehl-Neelsen, modified Kinyoun's), S: Sedimentation, NR: not reported

Table 3. Cryptosporidium species identified and subtyping.

Country	City	Species identified	%	Subtyping	Technique	Reference No.
Saudi Arabia	Dammam	*C. parvum*	30	-	S, BFM (E, I, AFS)	(46)
Kuwait	Kuwait	*C. parvum*	94	4 subtype allele families (IIa, IId. IIc, IIf)	SSU rRNA-based PCR-RFLP, 60-KD a glycoprotein-based DNA sequencing tool.	(6)
		C. hominis	5	3 subtype allele families (Id, Ie, Ib)		
Jordan	Irbid	*C. parvum*	37.3	-	DWM, SFC, BFM (AFS), DFA	(36)
Iraq	Basra	*C. parvum*	1	-	S, BFM (AFS)	(48)
Jordan	Bani-Kenanah	*C. parvum*	2	-	BFM (AFS), DFA	(47)

DFA: direct immunofluorescent assay, BFM: bright field microscope, RFLP: restriction fragment length polymorphism, E,I: eosin, iodine stain

munofluorescent assay as a screening tool, which calls into question the accuracy of the results. But in the one study where molecular tools such as PCR and RFLP were used, *C. hominis* (5%) and *C. parvum* (95%) were detected.[6]

The distribution of *Cryptosporidium* genotypes in the population is very different worldwide. Studies conducted in Peru, Thailand, Malawi, Uganda, Kenya, and South Africa showed a predominance of *C. hominis* in children or human immunodeficiency virus-positive adults.[60-64] In contrast, most of the patients investigated in Kuwait were infected with *C. parvum*. The only other region where *C. parvum* is more prevalent in humans than *C. hominis* is Europe, where several studies have shown a slightly higher prevalence of *C. parvum* over *C. hominis* in both immunocompetent and immunocompromised persons.[65-68]

The differences in the distribution of *Cryptosporidium* genotypes in humans are considered an indication of differences in infection sources.[68-70] Thus, the predominance of *C. parvum* in a population has been considered to be the result of zoonotic transmission. Indeed, even in areas with a high percentage of infections due to *C. parvum*, massive slaughter of farm animals during foot-and-mouth disease outbreaks can result in a reduction of the proportion of human infections due to *C. parvum*.[71]

Recent subtyping studies have shown that not all *C. parvum* infections in humans are the result of zoonotic transmission.[65,72,73] Among the *C. parvum* GP60 subtype families identified, alleles IIa and IIc (previously known as Ic) are the two most common types. The former has been identified in both humans and ruminants, whereas the latter has been seen only in humans.[65,73,74] In one of the studies included in this review two Kuwaiti children were infected with an allele IIc subtype strain, indicating that anthroponotic transmission of *C. parvum* occurs in Kuwait. Nevertheless, the low proportion of infections due to *C. hominis* suggests that anthroponotic transmission of cryptosporidiosis in Kuwait is probably not as important as in other countries. It is important to note that the other valid taxonomic species have not yet been detected in the region.

Returning to the outbreak of caprine cryptosporidiosis in the Sultanate of Oman, and to determine the reasons for it, it has been noted that the outbreak started during the cooler rainy season and this environmental factor might have imposed a stress responsible for the epizootic. However the rainy season may be associated with other factors, such as the enhanced survival of the organism in the environment or the seasonal variability of antibody levels observed in goats. Jonson et al.[75] observed an increase in incidence of enteric and respiratory diseases in goats during the rainy season in north-east Brazil and associated it with lower levels of serum antibodies recorded during the wet season than during the dry hot season. It has also been reported that goats who do not receive colostrum or are separated from their mothers and fed artificially, are more likely to develop cryptosporidial infections.[76-78] The kids in the Omani study were all suckled by their mothers and there was no indication that they had not received adequate quantities of colostrum. No immediate source of the infection was apparent. The water supply was not tested for cryptosporidia, but it seemed unlikely to have been the source because no other animals were affected and the treated water supply for the animals was the same as that for human consumption. It was a closed herd and the possibility that the infection was brought in from outside could therefore be ruled out.

In conclusion, future studies should focus predominantly on those in the age group <7 years, as this is consistent with the findings of previous studies in the region and elsewhere. The period of future studies should be at least 12 months to be able to detect any seasonal variation. Wherever possible, molecular techniques should be used for typing *Cryptosporidium* in order to provide valuable information about all circulating *Cryptosporidium* species and strains in the region. Finally, we recommend that physicians be aware of *Cryptosporidium* as a cause of diarrhea in children and that procedures for the diagnosis of this parasite be included in the routine diagnostic procedure for diarrheal stool specimens in all laboratories. This is particularly important since there is now an effective drug, nitazoxanide, available for therapy.[79]

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Characterization of rotavirus strains detected among children and adults with acute gastroenteritis in Gizan, Saudi Arabia

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ABSTRACT

الخلفية العلمية: تعتبر فيروسات الروتا من المسببات الأساسية للالتهابات المعوية في المملكة، واستشرافا لدخول التطعيمات المضادة للفيروس في البلاد تم عمل دراسة قصيرة لبحث سلالات الفيروس على حالات من الأطفال والبالغين المصابين بالتهابات معوية لعدد من المراجعين لمستشفيات المنطقة.

الطريقة: تم إجراء دراسة مقطعية في خمسة مستشفيات بمنطقة جازان، جمعت خلالها عينات براز من مرضى مصابين بإسهال حاد خلال الفترة من نوفمبر٢٠٠٤ إلى مارس ٢٠٠٥. تم الكشف على فيروس الروتا باستخدام طريقة المقايسة الإنزيمية المناعية (الإليزا) كما تم تعريف سلالات الفيروس باستخدام طريقة سلسلة تفاعلات إنزيم البلمرة (بي سي آر) وطريقة الرحلان الكهربائي (الإلكتروفوريسز).

النتائج: أظهرت الدراسة وجود الفيروس في 54 حالة من أصل 454 عينة جمعت بنسبة إصابة بلغت 12% في حالات تتراوح أعمارهم بين 15يوم إلى 20 سنة وسجلت أعلى نسبة إصابة بين الفئة العمرية (48–59شهر). بشكل عام تم تحديد نوعية 93% من العينات مع وجود النوع (ج1 ب8) في 89 % من الحالات كما عزل النوع (ج9ب8) والنوع (ج2ب4) في عينة لكل منهما.

٦خاتمة: هذه الدراسة هي الأولى في المملكة التي حددت النوع البائي للفيروس وعزل فيها لأول مرة النوع ج9. أظهرت الدراسة أن التوزيع السني الغير طبيعي لحالات الإصابة بفيروسات الروتا يستدعي دراسات مستقبلية بشكل أكبر توقعا لدخول التطعيم.

Objectives: To assess the circulating rotavirus strains among hospitalized children and adults in Gizan City.

Methods: This cross-sectional study was based in 5 hospitals in the Gizan area. Stool samples were collected between November 2004 and March 2005, from sequential patients with acute, dehydrating diarrhea. Rotavirus antigen was detected in stool by enzyme-linked immunosorbent assay. The diversity of rotavirus strains was investigated using electropherotying and reverse transcription-polymerase chain reaction amplification of the VP7 and VP4 genes (G and P genotyping).

Results: Rotavirus was detected in 54 of 454 (12%) subjects. The ages of those infected with rotavirus ranged from 15 days to 20 years, with a median age of 36 months. The highest rotavirus detection rate (24%) occurred in children aged 48-59 months. Overall, 50 (93%) of strains could be assigned both a G- and P-type; G1P[8] was the most frequently detected strain type (n=48, 89%) with one rotavirus each of G2P[4] and G9P[8].

Conclusion. Rotavirus strains circulating in Gizan would be well covered by current rotavirus vaccines. Rotavirus serotype G9 has been detected in Saudi Arabia for the first time. Continued surveillance of rotavirus strains is required.

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Rotavirus is recognized as a leading cause of severe gastroenteritis resulting in hospital admission and death in infants and young children worldwide.[1] Globally, an estimated 527,000 deaths each year are attributed to rotavirus infection among children under 5 years of age, and these deaths mainly occur in the developing countries.[2] Two rotavirus vaccines, namely

Rotarix, a human monovalent rotavirus vaccine (GSK Biologicals, Rixensart, Belgium) and RotaTeq, a human-bovine multivalent rotavirus vaccine (Merck Research Laboratories, Philadelphia, Pennsylvania, USA) have proven highly effective in preventing severe rotavirus gastroenteritis, and are now entering childhood immunization programmes in the Americas and in Europe.[3-5] Rotaviruses are triple-layered icosahedral particles and their genomes consist of 11 segments of double-stranded RNA. Based on epitopes on the inner capsid, rotaviruses are divided into 7 groups (A-G) but most human infections belong to group A. Rotaviruses are further classified according to the genetic and antigenic diversity of the 2 outer capsid proteins, VP4 and VP7. To date, 15 G serotypes and 26 P types have been defined.[6,7] Initially, these were defined as serotypes by panels of hyperimmune sera and monoclonal antibodies, but more recently genotyping has been possible using the reverse transcription - polymerase chain reaction (RT-PCR).[8,9] The G-serotype and G-genotype designations coincide, but those of P-types do not: the P-genotype is therefore indicated in a square bracket. As the segregation of VP7 genes and VP4 genes occurs independently, many G and P combinations are theoretically possible. Globally, however, there are 5 common rotavirus strains: these comprise serotypes G1, G3, G4, and G9 with P[8] VP4 specificity, and G2P[4] strains.[10,11] Rotavirus infection is a major cause of childhood morbidity in Saudi Arabia.[12] However, only 4 studies in Saudi have undertaken G-typing[13-16] and none has undertaken P-typing. It is considered important to describe the prevalent rotavirus strain types in a country before and after the introduction of rotavirus vaccines. In order to further investigate the diversity of circulating rotavirus strains in Saudi Arabia, we therefore conducted a 5-month study of rotavirus gastroenteritis in Gizan City.

Methods. This study was based in 5 hospitals in Gizan City and surrounding areas. The city is one of the largest cities in Saudi Arabia and is situated in the southern part of the kingdom. The climate is hot and humid for most of the year extending from April to October, followed by a milder season between December and February. Stool samples were collected from sequential patients with acute, dehydrating diarrhea who were either admitted to hospital, or who were given oral rehydration therapy as outpatients, from November 2004 to March 2005, thus covering both climatic seasons. All cases with acute watery diarrhea of less than 2 weeks were included. Information regarding age and gender of the patient, date of sample collection and whether the patient was treated as an in-patient or an out-patient were obtained. Any case without complete data was excluded. All stool samples were stored at -20°C until transported frozen to the Department of Medical Microbiology, University of Liverpool and stored at -80°C until further analysis. Ethical approval was given by the Research Ethics Committee, General Directory of Health Affairs in Gizan, Ministry of Health, Kingdom of Saudi Arabia. Verbal consent was obtained from the subjects.

Rotavirus antigen was detected in faeces by using the Rotaclone EIA kit (Meridian Diagnostics, Cincinnati, OH). The EIA positive faecal samples were suspended in phosphate buffered saline at a concentration of 10%. Suspensions were clarified by centrifugation and rotavirus double-stranded RNA (dsRNA) was extracted by using a guanidine isothiocyanate/silica method.[17] Rotavirus electropherotypes were determined by polyacrylamide gel electrophoresis (PAGE) of rotavirus dsRNA followed by silver staining as previously described.[18] Rotavirus genome profiles that could not be clearly categorized as short or long electropherotypes were labelled positive. Rotavirus G and P typing was determined using a hemi-nested, multiplex RT-PCR and used consensus and type-specific primers described previously for G-serotypes G1-5, G8, G9, G12 and P-types P[4], P[6], P[8], P[9] and P[10].[8,9,19] The RT-PCR products were resolved by electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualized by UV trans-illumination. Samples co-migrating with reference strains of known genotype were assigned to that genotype. Samples failing to G or P type were re-tested with additional G1 and P[8] typing primers NAC 9 and NAC 10.[20]

Statistical tests were performed by EPInfo version 6.0. The difference in prevalence between 2 groups was calculated by the Chi square test. A p value of <0.05 was considered significant.

Table 1 • Rotavirus detection rate by age.

Age (months)	No. of samples of gastroenteritis cases (%)	No. of rotavirus positive of age group (%)	Relative (%) distribution of rotavirus cases among those <5 years old (n=39)
0-5	25 (6)	2 (4)	5
6-11	27 (6)	4 (7)	10
12-23	73 (16)	9 (12)	23
24-35	60 (13)	9 (15)	23
36-47	64 (14)	8 (13)	21
48-59	29 (6)	7 (24)	18
≥60	176 (39)	15 (9)	
Total	454 (100)	54 (11.9)	

Table 2 - Electropherotypes and G and P types of rotaviruses from Gizan.

Electropherotype	P[8]		P[4]	P[NT]		Total (%)	
	G1	G9	G2	G1	GNT		
Long	35	1				36	(67)
Short							
Positive	6		1			7	(13)
Negative	6			3	1	10	(19)
Mixed	1					1	(2)
Total	48	1	1	3	1	54	

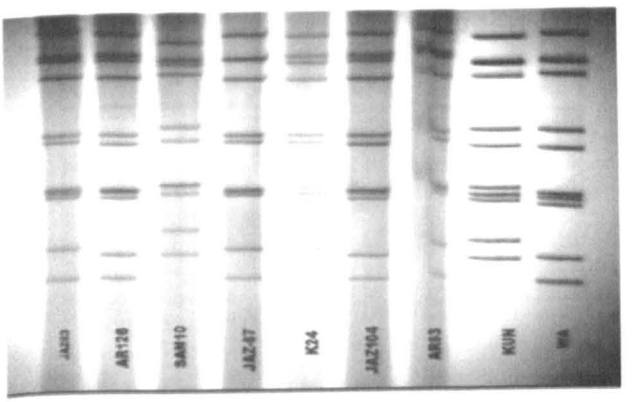

Figure 1 - Polyacrylamide gel electrophoresis and silver staining of rotavirus dsRNA genome. WA and KUN - Standard strains showing long electropherotype profile (strain WA), and short electropherotype profile (strain KUN). AR63 - Saudi field strain showing positive electropherotypes profiles G1P[8]. JAZ 104 and AR126 - Saudi field strains showing long electropherotypes profiles (L1) (G1P[8]). K24 and JAZ 63 - Saudi field strains showing long electropherotypes profiles (L2) (G1P[8]). JAZ 67 - Saudi field strain showing long electropherotypes profiles (L3) (G9P[8]). SAM10 - Saudi field strain showing short electropherotypes profiles (G2P[4]).

Results. A total of 54/454 (12%) specimens were positive for group A rotavirus, which was detected throughout the 5 months study period. The ages of subjects with rotavirus infection ranged from 15 days to 20 years (median age 36 months). The majority of rotavirus infections (39/54 [72%]) were identified among children under 5 years of age and nearly half (24/54 [44%]) were among children under age 3 years (**Table 1**). The highest rotavirus detection rate (7/29 [24%]) was observed in subjects aged 48-59 months, followed by children aged 60-72 months (11/53 [21%]), although differences in rotavirus detection rates did not reach statistical significance between age groups. Four (7%) of the 54 rotavirus infections occurred in subjects aged 9, 16, 19, and 20 years. Among the 54 rotaviruses identified, G- and P- types were determined for (53/54 [98%]) and (50/54 [93%]) rotavirus strains; 50 (93%) of rotaviruses could be assigned both a G- and a P-type. Of these, G1P[8] was the most common strain identified (48/54 [89%]), with one G2P[4] and one G9P[8] strain each detected. Fifteen of the G1 strains, and 14 of P[8] strains, were typeable only with the G1 primer NAC9 and P[8] primer NAC10, respectively. Ribonucleic acid bands could not be visualized for any of the 4 non-typeable samples using polyacrylamide gel electrophoresis. Overall, 36 (67%) of the 54 rotaviruses could be assigned an electropherotype (long); 7 were not interpretable (positive), one showed a mixed pattern and 10 were PAGE negative (**Table 2**). Among the long electropherotype strains 3 different patterns were identified (L1, L2, L3). The G1 strains produced patterns L1 and L2 and the G9 pattern L3 (**Figure 1**).

Discussion. The monthly prevalence of rotavirus gastroenteritis did not alter over the study period, which spanned the only seasonal changes in the region. Nevertheless, to properly assess seasonality at least 12 months surveillance would be needed. As expected, most cases of rotavirus gastroenteritis were in children under age 5 years. The slightly higher rate of rotavirus detection reported in the current study among children age >48 months is of interest, since the highest detection rates are normally seen in children <2 years of age.[1] Larger studies are required to determine whether these age-specific trends are real, or have occurred by chance. Fifteen (28%) of rotavirus infections were detected in subjects aged 5 years and older with the eldest being 9, 16, 19 and 20 years. These 4 infections were each with G1P[8] strains, which suggests that either immunity to this strain has waned over time, or perhaps that such strains were not circulating in Gizan whilst the subjects were infants. A similar situation has previously been described for serotype G1, G4, and G9 rotaviruses causing gastroenteritis in adults.[21-23] Detected strains of rotavirus were remarkably uniform, 89% of strains belonged to serotype G1P[8]. We identified only a single G2P[4] strain and one G9P[8] strain. However, 15 of the G1 strains were typeable only by using additional G1 primer NAC9.[20] In addition, a small number of strains could be neither G- nor P- typed (1 and 4 respectively). This could be explained by the presence of insufficient amounts of RNA in the non-typeable samples (these strains had negative PAGE profiles). A total of 22 studies of rotavirus gastroenteritis have been published from Saudi Arabia[12] with rotavirus prevalences ranging from 10-46%, with a median rotavirus detection rate of 30%. The only other study to be undertaken in Gizan

was from October 1983 to February 1984 which found a rotavirus prevalence of 16%.[24] Only 4 previous studies have provided data on G-serotypes in Saudi Arabia.[13-16] In each study, G1 was the predominant serotype with prevalences ranging from 39.6-53% and followed in decreasing order by G4, G3, and G2. This is the first report of G9P[8] rotavirus from Saudi Arabia and adds another country to the widening geographical distribution of this strain.[25]

Although this study is limited by a small sample size and short duration, it was described for the first time both P- and G- genotypes, and the occurrence of G9 rotavirus in Saudi Arabia. While available rotavirus vaccines would be expected to provide good coverage among currently circulating strains, continued rotavirus surveillance in the country is necessary to detect any changes in strain types pre- and post- rotavirus vaccine introduction.

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Cryptosporidium species causing acute diarrhoea in children in Antananarivo, Madagascar

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A 13-month study of children presenting with acute diarrhoeal disease at hospitals and rehydration clinics in Antananarivo, Madagascar, was undertaken between May 2004 and May 2005. Cryptosporidiosis accounted for diarrhoea in 12 (5.6%) of the 215 children investigated. Cases of cryptosporidiosis were detected only in the rainy season, and the median age of cases was 13.5 months (range = 1 day–27 months). As 11 of the cases of cryptosporidiosis were caused by *Cryptosporidium hominis* and only one by *C. parvum*, most of the cases were probably the result of anthroponotic transmission. GP60/45/15 gene polymorphisms indicated that the causative pathogens were of subtypes Ia, Id, Ie and IIc.

The protozoon parasites belonging to the genus *Cryptosporidium* are important causes of diarrhoeal disease in humans world-wide (Hart, 1999; Xiao and Ryan, 2004). As cryptosporidiosis can also affect a wide variety of animal species other than humans, however, there are both anthroponotic and zoonotic transmission cycles. *Cryptosporidium* was originally described in 1907, by Tyzzer (1907), as a sporozoan, named *C. muris*, found in the gastric glands of mice. Tyzzer (1912) subsequently described a similar coccidian in the murine small intestine, which he named *C. parvum*. Since then, many *Cryptosporidium* species have been named, largely based upon the parasites' morphological features and host species. With the introduction of molecular methods, principally sequencing of the 18S ribosomal RNA (rRNA) genes, more precise assignment to species became possible (Xiao and Ryan, 2004). Of the 13 species of *Cryptosporidium* currently accepted, *C. hominis*, *C. parvum*, *C. meleagiridis* and, to a lesser extent, *C. felis*, *C. canis* and *C. suis* are the most frequently detected in human infections, in developed and developing countries alike (Gatei *et al.*, 2003; Tumwine *et al.*, 2003; Xiao and Ryan, 2004). The diversity of infecting species appears to be greater in HIV-infected individuals than in the HIV-negative (Cama *et al.*, 2003).

Although *Cryptosporidium* spp. can infect the respiratory and biliary tracts, their major importance is in causing diarrhoeal disease (Hart, 1999). In developing countries cryptosporidiosis occurs predominantly in children under 5 years of age, with the highest prevalence in infants in their second year of life (Tumwine *et al.*, 2003; Gatei *et al.*, 2006*b*). *Cryptosporidium* spp. are usually the fourth or fifth commonest cause of diarrhoeal disease in children (after enteropathic viruses such as rotavirus, norovirus and

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astrovirus), and are responsible for 5%–12% of all cases in the developing countries of Africa, Asia and South America (Hart, 1999). Cryptosporidiosis has a world-wide distribution, having been detected in every country where it has been sought (Hart, 1999).

Madagascar is a large island archipelago in the Indian Ocean, off the south–eastern coast of Africa. It is the fourth largest island in the world and home to 5% of the world's plant and animal species (80% of which are unique to Madagascar). It has a population of around 18.6 million people. Although it has an annual population growth rate of 2.6%, about 85,000 of its young children (aged <5 years) die each year. There are few published studies of diarrhoeal disease from Madagascar and none at all on cryptosporidiosis on the island.

SUBJECTS AND METHODS

Stool samples were collected from children attending rehydration clinics or admitted to hospital with acute dehydrating diarrhoea in Antananarivo, Madagascar's capital city. The study was conducted from the Institut Pasteur, in Antananarivo, from May 2004 to May 2005 inclusive. The study protocol was approved by the Ethical Review Board of the Institut Pasteur de Madagascar and the National Ethical Committee of Madagascar, and the investigation was funded internally by the University of Liverpool's Department of Medical Microbiology. The faecal samples were frozen without additives and stored at −80°C until transported frozen to Liverpool for analysis.

All samples were screened for norovirus and astrovirus by reverse-transcriptase PCR and for rotavirus by ELISA, as described previously (Belliot *et al.*, 1997; Cunliffe *et al.*, 2001; Papaventsis *et al.*, 2007). Unconcentrated fixed faecal smears were stained by the modified Ziehl–Neelsen method and examined, by bright-field microscopy, for *Cryptosporidium* oocysts (Casemore and Roberts, 1993). In addition, all stool suspensions were tested for the presence of *Cryptosporidium* antigen using a commercial enzyme immuno-assay (ProSpecT *Cryptosporidium* microplate assay: Alexon-Trend, Ramsay, MN). Bacterial enteropathogens were not sought as these require culture and the viability of such pathogens is variable following their freezing and transport. Each sample found positive for *Cryptosporidium* antigen was also screened by negative-stain electron microscopy (Baxby *et al.*, 1984).

Each confirmed *Cryptosporidium*-positive sample was then subjected to genotype analysis. After oocyst lysis, by six cycles of freezing (at −80°C for 30 min) and thawing (at 80°C for 15 min), DNA was extracted using a QIAamp DNA stool mini kit (QIAGEN, Crawley, U.K.), precipitated in absolute ethanol, and stored at −20°C until used. Analysis of restriction-fragment length polymorphisms (RFLP), sequencing of the PCR-amplified 18S rDNA gene, and sequencing of *Hsp70* gene amplicons were used to identify the *Cryptosporidium* to species (Xiao *et al.*, 2001); the restriction endonucleases used in the RFLP analysis were *Ssp*I and *Vsp*I (Boehringer Mannheim, Mannheim, Germany). For assignment to subtype, the Gp15/45/60 gene from each sample was amplified by PCR and sequenced (Sulaiman *et al.*, 2005; Gatei *et al.*, 2006*a*).

RESULTS

Overall, 215 stool samples — one each from 215 children — were screened for the presence of *Cryptosporidium* oocysts and antigen. Of the 215 children studied in this way, 129 (60%) were male and 85%, 77%, 43% were aged <3 years, <2 years and <1 year, respectively, with 3% considered neonates. The median age of the children was 20 months (range=1 day–16 years). Although 16 samples were found positive

TABLE 1. *The relative importance of* Cryptosporidium *species as enteropathogens among the children with acute dehydrating diarrhoea*

Pathogen	No. and (%) of children: Investigated*	Found infected	Median aged of infected children (months)
Astrovirus	237	5 (2.1)	10
Cryptosporidium sp.	215	12 (5.6)	13.5
Norovirus	237	14 (5.9)	18
Rotavirus	258	109 (42.2)	10

*Some faecal samples were too small to be tested for all four pathogens.

for *Cryptosporidium* antigen (four giving optical densities close to the kit manufacturer's cut-off for antigen positivity), only 12 were confirmed to be *Cryptosporidium*-positive by light and electron microscopy and PCR amplification; the other four (the children who gave the lowest optical densities among the antigen-positive samples) were classified as false-positives. Thus, 12 (5.6%) of the 215 children checked, each of whom had acute dehydrating diarrhoea, were found infected with *Cryptosporidium* spp. This compares with prevalences of 42% for rotavirus, 6% for norovirus and 2.5% for astrovirus (see Table 1). None of the children with cryptosporidiosis were co-infected with any of the viral enteropathogens. Seven (58%) of the children with cryptosporidiosis were male. The ages of the *Cryptosporidium*-positive children ranged from 1 day to 27 months but 10 (83%) were in their second year of life (Table 2). The median age of the children with cryptosporidiosis (13.5 months) was similar to those of the children infected with rotavirus (10 months) or astrovirus (10 months) and insignificantly lower than the median age of the children found to be infected with norovirus (18 months; $P=0.12$).

The cases of cryptosporidiosis were not evenly distributed throughout the year: four (33%) presented in November, three (25%) in December, four (33%) in February and one in March (see Table 2). No cases were detected in the remaining months. The hot and rainy season in Madagascar usually runs from November to the end of April. Among

TABLE 2. *The age and gender of the 12 children found infected with* Cryptosporidium *and the species and genotype of their* Cryptosporidium

Patient	Age	Gender	Month of presentation	Species	Subtype (Gp60)
DR0014	14 months	Female	November 2004	*C. hominis*	IaA22R3
DR0031	23 months	Male	November 2004	*C. hominis*	IdA15G1
DC2049	13 months	Male	November 2004	*C. hominis*	IdA15G1
DR0037	8 months	Male	November 2004	*C. hominis*	NA
DT1025	12 months	Male	December 2004	*C. hominis*	I
DG6023	15 months	Male	December 2004	*C. hominis*	IaA22R3
DR0063	14 months	Male	December 2004	*C. hominis*	NA
DR0072	13 months	Male	February 2005	*C. hominis*	IdA15G1
DT1038	20 months	Female	February 2005	*C. hominis*	IeA11G3T3
DR0077	14 months	Female	February 2005	*C. parvum*	IIcA5G3
DR0079	27 months	Female	February 2005	*C. hominis*	IaA22R3
DR1060	1 day	Female	March 2005	*C. hominis*	IdA15G1

NA, Not amplified.

the children with acute dehydrating diarrhoea who were investigated, the prevalence of cryptosporidiosis was higher among the 175 who presented in the hot rainy season than among the 40 who presented at other times (6.9% *v.* 0%), although this difference did not reach statistical significance ($P=0.088$).

Analysis of the 18S rDNA RFLP patterns and the sequencing of the 18S rDNA and *Hsp70* PCR amplicons indicated that all but one of the *Cryptosporidium*-positive children were infected with *C. hominis*; the other child appeared to be infected with *C. parvum*. As expected, all eight *C. hominis* isolates successfully subtyped were of Gp60 type I whereas the single *C. parvum* isolate was found to be type II (Table 2). All three *C. hominis* strains identified as Ia were IaA22R3 and all three identified as Id were IdA15G1. Another *C. hominis* strain was IeA11G3T3, and the *C. parvum* was IIcA5G3 (Table 2).

DISCUSSION

As this is the first study of cryptosporidiosis in Madagascar, no other relevant data from the island are available for comparison. In the present, 13-month study of children with acute dehydrating diarrhoea, the prevalence of cryptosporidiosis was found to be 5.6% (12/215). Cases were defined as those who were positive by each of the diagnostic tests used (antigen detection, light and electron microscopy and PCR). Four children were antigen-positive but at an optical density close to the kit manufacturer's cut-off for positivity, and all four were negative by each of the other diagnostic tests and thus considered false-positives. The specificity of the commercial antigen-detection ELISA in this study was therefore 98% (203/297), which is at the upper end of the range (89%–99%) reported by others (Rosenblatt and Sloan, 1993; Aarnaes *et al.*, 1994; Geurden *et al.*, 2006).

In terms of its relative importance as an enteropathogen, *Cryptosporidium* was more prevalent than astrovirus (2.1%), equivalent to norovirus (6%) but significantly ($P<0.001$) less important than rotavirus (42%). In most hospital-based surveys, *Cryptosporidium* spp. have been found to be among the five commonest causes of diarrhoeal disease (Hart, 1999). Among the cases of acute diarrhoeal disease in African children investigated up until 1998, *Cryptosporidium* species had a reported median prevalence of 8.7% (Hart, 1999). In the studies of at least 1 year's duration conducted since 1998, the recorded prevalences have ranged from 4% in Kenya (Gatei *et al.*, 2006*b*) to 8.5% in Uganda (Tumwine *et al.*, 2003) and 14.8% in Guinea-Bissau (Perch *et al.*, 2001). Although Adjei *et al.* (2004) reported an even higher prevalence (of 27.8%) in children in Ghana, their relatively short survey period may simply have coincided with a seasonal peak in prevalence.

In Madagascar, all the detected cases of cryptosporidiosis presented in November, December, February or March, that is, during the rainy season in Antananarivo. In studies in Gaza (Sallon *et al.*, 1991), Kampala in Uganda (Tumwine *et al.*, 2003) and Lusaka in Zambia (Nchito *et al.*, 1998), cases of cryptosporidiosis also peaked in the rainy season. In contrast, however, in their large study of 4899 patients from different regions of Kenya, Gatei *et al.* (2006*b*) found that prevalence peaked between November and February — typically the driest season of the year in Kenya, following the short rains.

The median age of children with cryptosporidiosis in Madagascar was 13.5 months, although this value was skewed by the neonate who was found infected at an age of 1 day. Although unusual, an incubation period of 1 day or less has been reported previously (Jokipii and Jokipii, 1986; Millard *et al.*, 1994). Unfortunately, no information is available about the mother of the infected baby. All but one of the other

cases detected were children in their second year of life. This age-specific trend in prevalence is different from that reported in Egypt (Abdel-Messih *et al.*, 2005), Uganda (Tumwine *et al.*, 2003) and Tanzania (Cegielski *et al.*, 1999), where the prevalence of cryptosporidiosis was found to be highest in infants in their first year of life. In the U.K. (Baxby and Hart, 1986), Gaza (Sallon *et al.*, 1994) and Kenya (Gatei *et al.*, 2006*b*), however, prevalences have also been found to peak in children aged 12–24 months. It is unclear why such geographical differences in the age-related trends in prevalence should occur.

Until 2002, the predominant species of *Cryptosporidium* infecting humans was reported to be *C. parvum*. This taxon was subdivided into *C. parvum* genotype 1 (human) and genotype 2 (bovine). *Cryptosporidium parvum* genotype 1 is now recognised as a 'new', anthroponotic species, *C. hominis*, whereas *C. parvum* genotype 2 is now known simply as *C. parvum*, a parasite that may have zoonotic or anthroponotic cycles (Morgan-Ryan *et al.*, 2002; Xiao and Ryan, 2004). Most (92%) of the *Cryptosporidium* isolates detected in the present study were *C. hominis*. This anthroponotic species has also been found to predominate in Uganda (74% *C. hominis*; Tumwine *et al.*, 2005) and Kenya (87% *C. hominis*; Gatei *et al.*, 2006*b*). The predominance of *C. hominis* may be related to the urban nature of the present study. Based on DNA sequences of a fragment of the Gp60/45/15 glycoprotein gene, three subtype families of *C. hominis* (Ia, Id and Ie) were detected in the present study and the *C. parvum* isolate was found to belong to the IIc subtype family. Interestingly, *C. parvum* of subtype family IIc has only been found in human infections (Alves *et al.*, 2006). The three children infected with the same subtype family of *C. hominis* (IdA15G1) were unrelated (data not shown) and their infections were detected in different months. Although, in Portugal, Alves *et al.* (2006) found that the *C. hominis* subtype family responsible for most human cases of cryptosporidiosis was Ib, this subtype has not been detected in Madagascar. In India, however, Gatei *et al.* (2007) detected *C. hominis* of the Ia, Ib, Id and Ie subtype families, which include each of the subtype family alleles detected, in Madagascar, in the present study.

Cryptosporidiosis is an important cause of acute and chronic diarrhoea. More information on its prevalence, molecular epidemiology and impact is urgently required. Knowledge of the prevalence and impact of cryptosporidiosis in different parts of the world would be especially useful now that a safe and effective anti-cryptosporidial drug, nitazoxanide, is available (White, 2003).

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Cryptosporidiosis in Gizan Area, Saudi Arabia

Medical Microbiology and G-U Medicine

Mohammed Areeshi, C.A. Hart and N.J. Beeching

Introduction :

Cryptosporidiosis is an acute diarrhoeal disease caused by the protozoan *Cryptosporidium* spp.

Cryptosporidium species are apicomplexan parasites that infect the microvillus border of the gastrointestinal epithelium of a wide range of vertebrate hosts, including humans.

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.

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.

The prevalence of *Cryptosporidium* infection in children in various studies ranges from 2% - 40% and among HIV/ AIDS patients from 14% - 50% with developing countries having the higher prevalence.

Globally, *Cryptosporidium* has now been recognized 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.

Map of Saudi Arabia

Study area

This study was undertaken in Gizan Area which is located in the south west of the Kingdom of Saudi Arabia. It is an important port on the Red Sea. It has a hot and humid climate all year-round.

The samples of the study were collected from five different parts of the area namely: Al-Ardah, Al-Khobah, Samtah, Abu-Areesh and Gizan (Capital city).

Map of Gizan Area, Saudi Arabia

Study population:

In humans, the study group included children from 0-6 years of age attending a hospital or a clinic as inpatients or outpatients with diarrhoea without gastroenteritis. In addition to asymptomatic school children (5 years old) who were used as controls.

In animals, the study group included animals from 0-2 years of age targeting the diarrhoeal cases. The samples were collected from cows, goats and sheeps.

OBJECTIVES

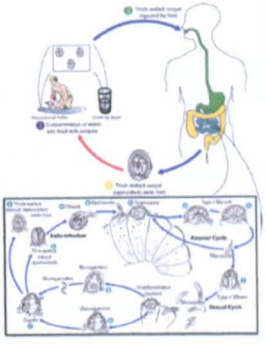
The main objectives of the study were to determine the following:

-The relative importance of *Cryptosporidium* as a cause of children diarrhoea among other enteropathogens.

-The distribution and genotypes of different *Cryptosporidium* species.

-The age specific prevalence of cryptosporidiosis.

-The relationship between *Cryptosporidium* in humans and domestic animals in the south west of Saudi Arabia.



Cryptosporidium parvum life cycle.

Methodology: -

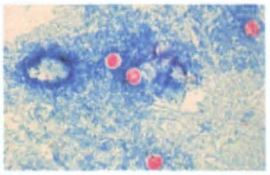
795 stool samples were collected (454 from patients, 62 from asymptomatic school children (controls) and 279 from animals including goats, sheeps and cows) between October 2004 and February 2005.

All samples were screened by bright field microscopy looking for *Cryptosporidium* oocysts using a modified Ziehl-Neelsen (ZN) and Safranin Methylene Blue (SM-B) staining techniques.

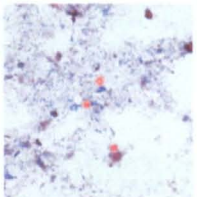
Enzyme Linked Immunosorbent Assay (ELISA) test was performed on all samples to confirm the positivity by using ProSpoect *Cryptosporidium* microplate Assay kit.

DNA was extracted using QIAMP DNA extraction kit.

18S rRNA gene was targeted for the diagnosis of *Cryptosporidium* followed by Restriction Fragment Length Polymorphism (RFLP) to identify the species.



Cryptosporidium oocysts stained with Ziehl Neelsen with 40x magnification

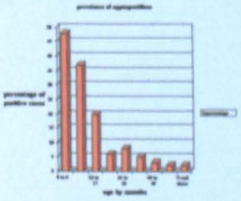


Cryptosporidium oocysts stained with Safranin Methyline Blue with 40x magnification

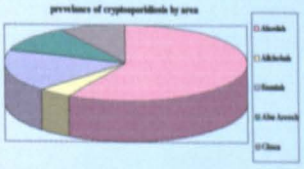
Results:

Prevalence:

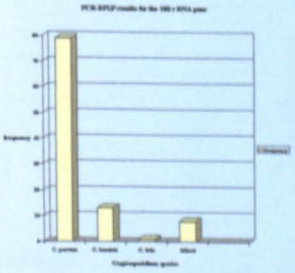
Cryptosporidium oocysts was detected in 45 samples (9.9%) out of 454 of the human samples and in one animal sample (0.36 %) out of 279 of the animal samples. No oocysts were detected among the school children (controls).



Cryptosporidium infection was more common (71.1%) in children less than two years of age ($p<0.01$), and about half (48.9%) of the detected positives were among children in the first year of life ($p<0.01$). The highest numbers of cases were observed in patents (0-5) months of age (48%).



The highest prevalence of cryptosporidiosis was in Al-Ardah (57.8%) followed by Samtah (17.8%), while the lowest prevalence was in Al-Khobah (4.4%).



The most frequent species identified in Gizan was *C. parvum* with 24/43 (55.8%) followed by *C. hominis* with 10/43 (23.3%). *C. felis* was identified in one sheep sample (2.3%) while 8 (18.6%) samples contained a mixture of the two species, *C. parvum* and *C. hominis*

Conclusion: It has been concluded that:

-There was a significant relationship between young age and cryptosporidiosis and the infection was more common in children less than two years of age.

-the 18S rRNA gene target is an important tool for the precise identification of *Cryptosporidium* species especially due to wide application of a single set of primers for the different species.

-Physicians should be aware of *Cryptosporidium* as a cause of diarrhoea in children and that procedures for the diagnosis of this parasite be included in the routine diagnostic procedure for diarrhoeal stool specimens in all laboratories

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1: AB369993. Reports Cryptosporidium p...[gi:161410620] Links

Features Sequence

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LOCUS       AB369993                842 bp    DNA     linear   INV 01-DEC-2007
DEFINITION  Cryptosporidium parvum gene for 18S ribosomal RNA, isolate: Md
            1055.
ACCESSION   AB369993
VERSION     AB369993.1  GI:161410620
KEYWORDS    .
SOURCE      Cryptosporidium parvum
  ORGANISM  Cryptosporidium parvum
            Eukaryota; Alveolata; Apicomplexa; Coccidia; Eucoccidiorida;
            Eimeriorina; Cryptosporidiidae; Cryptosporidium.
REFERENCE   1
  AUTHORS   Areeshi,M.Y., Hart,C.A. and Beeching,N.J.
  TITLE     Cryptosporidiosis in two regions of Saudi Arabia
  JOURNAL   Published Only in Database (2007)
REFERENCE   2  (bases 1 to 842)
  AUTHORS   Areeshi,M.Y.
  TITLE     Direct Submission
  JOURNAL   Submitted (29-NOV-2007) Contact:Mohammed Y Areeshi The University
            of Liverpool, Infection and Host Defence; 8th Floor Duncan
            Building, Daulby Street, Liverpool L693GA, United Kingdom
FEATURES             Location/Qualifiers
     source          1..842
                     /organism="Cryptosporidium parvum"
                     /mol_type="genomic DNA"
                     /isolate="Md 1055"
                     /isolation_source="diarrhetic human child"
                     /db_xref="taxon:5807"
                     /country="Saudi Arabia"
                     /collection_date="20-Oct-2004"
                     /collected_by="Mohammed Areeshi"
     rRNA            1..842
                     /product="18S ribosomal RNA"
ORIGIN
        1 tgtgaaaggg tgtgtttttt tagtgataag raysamtagt mattggtgac tcataataac
       61 tttacggatc acattaaatg tgacatatca ttcaagtttc tgacctatgt tccttagacg
      121 gtagggtatt ggcctaccgt ggcaatgacg ggtaacgggg aattagggtt cgattccgga
      181 gagggagcct gagaaacggc taccacatct aaggaaggca gcaggcgcgc aaattaccca
      241 atcctaatac agggaggtag tgacaagaaa taacaataca ggacttttta gttttgtaat
      301 tggaatgagt taagtataaa ccccttaca agtatcaatt ggagggcaag tctggtgcca
      361 gcagccgcgg taattccagc tccaatagcg tatattaaag ttgttgcagt taaaaagctc
      421 gtagttggat ttctgttaat aatttatata aaatattttg atgaatattt atataatatt
      481 aacataattc atattactat atattttagt atatgaaatt ttactttgag aaaattagag
      541 tgcttaaagc aggcatatgc cttgaatact ccagcatgga ataatattaa agatttttat
      601 ctttcttatt ggttctaaga taagaataat gattaatagg gacagttggg ggcatttgta
      661 tttaacagtc agaggtgaaa ttcttagatt tgttaaagac aaactaatgc gaaagcattt
      721 gccaaggatg ttttcattga tcaagaacga aagttagggg atcgaagacg atcagatacc
      781 gtcgtagtct tacataaact atgccaacta gagatsagag gggggcccc tactccctca
      841 aa
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1: AB369994. Reports Cryptosporidium h...[gi:161410621] Links

Features Sequence

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LOCUS       AB369994                983 bp    DNA     linear   INV 01-DEC-2007
DEFINITION  Cryptosporidium hominis gene for 18S ribosomal RNA, isolate: Ar108.
ACCESSION   AB369994
VERSION     AB369994.1  GI:161410621
KEYWORDS    .
SOURCE      Cryptosporidium hominis
  ORGANISM  Cryptosporidium hominis
            Eukaryota; Alveolata; Apicomplexa; Coccidia; Eucoccidiorida;
            Eimeriorina; Cryptosporidiidae; Cryptosporidium.
REFERENCE   1
  AUTHORS   Areeshi,M.Y., Hart,C.A. and Beeching,N.J.
  TITLE     Cryptosporidiosis in two regions of Saudi Arabia
  JOURNAL   Published Only in Database (2007)
REFERENCE   2  (bases 1 to 983)
  AUTHORS   Areeshi,M.Y.
  TITLE     Direct Submission
  JOURNAL   Submitted (29-NOV-2007) Contact:Mohammed Y Areeshi The University
            of Liverpool, Infection and Host Defence; 8th Floor Duncan
            Building, Daulby Street, Liverpool L693GA, United Kingdom
FEATURES             Location/Qualifiers
     source          1..983
                     /organism="Cryptosporidium hominis"
                     /mol_type="genomic DNA"
                     /isolate="Ar108"
                     /isolation_source="diarrhetic human child"
                     /db_xref="taxon:237895"
                     /country="Saudi Arabia"
                     /collection_date="15-Nov-2004"
                     /collected_by="Mohammed Areeshi"
     rRNA            1..983
                     /product="18S ribosomal RNA"
ORIGIN
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       61 tttggcctat tctggggggc ggcatttata tcataaaatt tctgtgggga agagagacca
      121 ctctgagaaa tttctcggaa aactctttca agggggttgt ttttttttttg gaaaaccaat
      181 ataattggtg actcataata actctacgga tcacaattaa tgtgacatat cattcaagtt
      241 tctgacctat cagctttaga cggtagggta ttggcctacc gtggcaatga cgggtaacgg
      301 ggaattaggg ttcgattccg gagagggagc ctgaaaaacg gctaccacat ctaaggaagg
      361 cagcaggcgc gcaaattacc caatcctaat acagggaggt agtgacaaga aataacaata
      421 caggactttt tggttttgta attggaatga gttaagtata aacccctttа caagtatcaa
      481 ttggagggca agtctggtgc cagcagccgc ggtaattcca gctccaatag cgtatattaa
      541 agttgttgca gttaaaaagc tcgtagttgg atttctgtta ataatttata taaaatattt
      601 tgatgaatat ttatataata ttaacataat tcatattact attttttttt ttagtatatg
      661 aaattctact ttgagaaaat tagagtgctt aaagcaggca tatgccttga atactccagc
      721 atggaataat attaaagatt tttatctttt ttattggttc taagataaga ataatgatta
      781 atagggactg ttgggggcat ttgtatttaa cagtcagagg tgaaattctt agatttgtta
      841 aagacaaact aatgcgaaag catttgccaa ggatgttttc attaatcaag aacgaaagtt
      901 aggggatcga agacgatcag ataccgtcga agtcttaacc ataaactatg ccaactagag
      961 atgagttgtc tctatactct ata
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1: AB369995. Reports Cryptosporidium p...[gi:161410622] Links

Features Sequence

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LOCUS       AB369995                 291 bp    DNA     linear   INV 01-DEC-2007
DEFINITION  Cryptosporidium parvum GP60 gene for 60 kD glycoprotein, partial
            cds, isolate: Md 1055.
ACCESSION   AB369995
VERSION     AB369995.1  GI:161410622
KEYWORDS    .
SOURCE      Cryptosporidium parvum
  ORGANISM  Cryptosporidium parvum
            Eukaryota; Alveolata; Apicomplexa; Coccidia; Eucoccidiorida;
            Eimeriorina; Cryptosporidiidae; Cryptosporidium.
REFERENCE   1
  AUTHORS   Areeshi,M.Y., Hart,C.A. and Beeching,N.J.
  TITLE     Cryptosporidiosis in two regions of Saudi Arabia
  JOURNAL   Published Only in Database (2007)
REFERENCE   2  (bases 1 to 291)
  AUTHORS   Areeshi,M.Y.
  TITLE     Direct Submission
  JOURNAL   Submitted (29-NOV-2007) Contact:Mohammed Y Areeshi The University
            of Liverpool, Infection and Host Defence; 8th Floor Duncan
            Building, Daulby Street, Liverpool L693GA, United Kingdom
FEATURES             Location/Qualifiers
     source          1..291
                     /organism="Cryptosporidium parvum"
                     /mol_type="genomic DNA"
                     /isolate="Md 1055"
                     /isolation_source="diarrhetic human child"
                     /db_xref="taxon:5807"
                     /country="Saudi Arabia"
                     /collection_date="25-Nov-2004"
                     /collected_by="Mohammed Areeshi"
     gene            <1..>291
                     /gene="GP60"
     CDS             <1..>291
                     /gene="GP60"
                     /codon_start=3
                     /product="60 kD glycoprotein"
                     /protein_id="BAF94157.1"
                     /db_xref="GI:161410623"
                     /translation="SSSSSSXSSSSSSSSSSSSSSTSTVAPTPKKERTGEEVGNPGSE
                     GQDGKGDTEETEENQTESTVSQNTPAQTEGTTTETTEAAPKKECGTSFVMWS"
ORIGIN
        1 tctcatcatc atcatcatca tsatcatcat catcatcatc atcatcgtca tcatcatcat
       61 catcaacatc gactgtagca ccaactccaa agaaagaaag aactggagag gaagtaggta
      121 atccaggttc tgaaggtcag gacggtaaag gagacactga agaaacagaa gaaaatcaga
      181 ccgagagtac tgtttctcaa aatactccag ctcaaactga aggcacaact accgaaacca
      241 cagaagctgc tccaaagaaa gagtgcggta cttcatttgt aatgtggtcg a
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1: AB369996. Reports Cryptosporidium h...[gi:161410624] Links

Features Sequence

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LOCUS       AB369996                333 bp    DNA     linear   INV 01-DEC-2007
DEFINITION  Cryptosporidium hominis GP60 gene for 60 kD glycoprotein, partial
            cds, isolate: Ar108.
ACCESSION   AB369996
VERSION     AB369996.1  GI:161410624
KEYWORDS    .
SOURCE      Cryptosporidium hominis
  ORGANISM  Cryptosporidium hominis
            Eukaryota; Alveolata; Apicomplexa; Coccidia; Eucoccidiorida;
            Eimeriorina; Cryptosporidiidae; Cryptosporidium.
REFERENCE   1
  AUTHORS   Areeshi,M.Y., Hart,C.A. and Beeching,N.J.
  TITLE     Cryptosporidiosis in two regions of Saudi Arabia
  JOURNAL   Published Only in Database (2007)
REFERENCE   2  (bases 1 to 333)
  AUTHORS   Areeshi,M.Y.
  TITLE     Direct Submission
  JOURNAL   Submitted (29-NOV-2007) Contact:Mohammed Y Areeshi The University
            of Liverpool, Infection and Host Defence; 8th Floor Duncan
            Building, Daulby Street, Liverpool L693GA, United Kingdom
FEATURES             Location/Qualifiers
     source          1..333
                     /organism="Cryptosporidium hominis"
                     /mol_type="genomic DNA"
                     /isolate="Ar108"
                     /isolation_source="diarrhetic human child"
                     /db_xref="taxon:237895"
                     /country="Saudi Arabia"
                     /collection_date="05-Jan-2005"
                     /collected_by="Mohammed Areeshi"
     gene            <1..>333
                     /gene="GP60"
     CDS             <1..>333
                     /gene="GP60"
                     /codon_start=3
                     /product="60 kD glycoprotein"
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                     /db_xref="GI:161410625"
                     /translation="SSSSTTPAPASKNVREAEGSEEKDSEEKDSEEKGSEEGSQTPAS
                     PGGGGVSEGDTQGDSKGDGVSSDENQSQGGDATPGSSTQTQATEKEPGSSEATPKEEC
                     GTSFVMWF"
ORIGIN
        1 catcatcgtc gtcaacaacc ccagcaccag cttcaaagaa tgtaagagaa gcagaaggca
       61 gtgaagaaaa ggacagcgaa gaaaaggaca gtgaagaaaa gggcagtgaa gaaggtagcc
      121 aaactcccgc tagtcctgga ggtggagggg tgagtgaagg agatactcaa ggtgactcta
      181 aaggagacgg agttagttca gatgagaacc aaagtcaagg tggggacgct actcccggat
      241 ctagcaccca aactcaagct actgaaaaag aacccggatc ttcagaagct actccaaagg
      301 aagagtgcgg tacttcattt gtaatgtggt tcg
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1: AB369997. Reports Cryptosporidium p...[gi:161410626] Links

Features Sequence

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LOCUS       AB369997                927 bp    DNA     linear   INV 01-DEC-2007
DEFINITION  Cryptosporidium parvum HSP70 gene for 70 kD heat shock protien,
            partial cds, isolate: Md 1055.
ACCESSION   AB369997
VERSION     AB369997.1  GI:161410626
KEYWORDS    .
SOURCE      Cryptosporidium parvum
  ORGANISM  Cryptosporidium parvum
            Eukaryota; Alveolata; Apicomplexa; Coccidia; Eucoccidiorida;
            Eimeriorina; Cryptosporidiidae; Cryptosporidium.
REFERENCE   1
  AUTHORS   Areeshi,M.Y., Hart,C.A. and Beeching,N.J.
  TITLE     Cryptosporidiosis in two regions of Saudi Arabia
  JOURNAL   Published Only in Database (2007)
REFERENCE   2  (bases 1 to 927)
  AUTHORS   Areeshi,M.Y.
  TITLE     Direct Submission
  JOURNAL   Submitted (29-NOV-2007) Contact:Mohammed Y Areeshi The University
            of Liverpool, Infection and Host Defence; 8th Floor Duncan
            Building, Daulby Street, Liverpool L693GA, United Kingdom
FEATURES             Location/Qualifiers
     source          1..927
                     /organism="Cryptosporidium parvum"
                     /mol_type="genomic DNA"
                     /isolate="Md 1055"
                     /isolation_source="diarrhetic human child"
                     /db_xref="taxon:5807"
                     /country="Saudi Arabia"
                     /collection_date="20-Jan-2005"
                     /collected_by="Mohammed Areeshi"
     gene            <1..>927
                     /gene="HSP70"
     CDS             <1..>927
                     /gene="HSP70"
                     /codon_start=1
                     /product="70 kD heat shock protien"
                     /protein_id="BAF94159.1"
                     /db_xref="GI:161410627"
                     /translation="IGWWSTRIPKVQALIQEFFNGKEPCKAINPDEAVAYGAAVQAAI
                     LNGEQSSAVQDLLLLDVAPLSLGLETAGGVMTKLIERNTTIPAKKTQVFTTYADNQSG
                     VLIQVYEGERAMTKDNHLLGKFHLDGIPPAPRGVPQIEVTFDIDANGILNVSAVDKST
                     GKSSKITITNDKGRLSKDDIERMVNDAEKYKGEDEQNRLKIEAKNSLENYLYNMRNTI
                     QEPKVKEKLSQSEIDEAEKKIKDALDWLEHNQTAEKDEFEHQQKEIETHMNPLMMKIY
                     SAEGGMPGGMPGGMPGGMPGGMPGGMPGGMPGG"

ORIGIN
        1 attggttggt ggtctacacg tattccaaag gttcaggcct tgattcagga attctttaac
       61 ggtaaagagc catgcaaagc aatcaatcca gacgaagctg ttgcttatgg tgctgctgta
      121 caagctgcta tcttaaatgg tgagcaatcc tctgccgtac aggatctctt attattggat
      181 gttgctccat tatcactcgg tttagaaact gctggtggtg ttatgaccaa gcttattgaa
      241 cgtaatacaa ctatcccagc aaagaagaca caagtcttca ctacttatgc tgataaccag
      301 agtggtgtct tgatccaagt ttatgagggt gagagagcca tgactaagga taaccatctc
      361 ctcggaaagt tccatcttga tggtattcca ccagcaccaa gaggtgtacc acaaattgaa
      421 gtcacctttg atattgatgc taatggtatc ttgaatgtgt ctgctgttga taagagtact
      481 ggtaagagca gcaagatcac tattactaac gataagggta gattatcaaa ggacgatatt
      541 gaacgtatgg ttaatgatgc tgagaaatac aagggtgagg atgagcagaa cagacttaag
      601 attgaggcta agaactcttt ggagaactac ctctataaca tgaggaacac catccaagaa
      661 ccaaaggtta aggaaaagct ttctcaatct gaaattgatg aggctgagaa gaagatcaag
      721 gatgctcttg actggctcga gcacaaccaa actgctgaaa aggacgagtt tgaacatcaa
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     781 caaaaggaga ttgaaactca tatgaatcca ctcatgatga agatctactc tgctgagggt
     841 ggtatgccag gtggaatgcc aggtggtatg ccaggcggta tgccaggtgg aatgccaggt
     901 ggtatgccag gtggaatgcc aggcggt
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1: AB369998. Reports Cryptosporidium h...[gi:161410628] Links

Features Sequence

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LOCUS       AB369998                829 bp    DNA     linear   INV 01-DEC-2007
DEFINITION  Cryptosporidium hominis HSP70 gene for 70 kD heat shock protien,
            partial cds, isolate: Ar108.
ACCESSION   AB369998
VERSION     AB369998.1  GI:161410628
KEYWORDS    .
SOURCE      Cryptosporidium hominis
  ORGANISM  Cryptosporidium hominis
            Eukaryota; Alveolata; Apicomplexa; Coccidia; Eucoccidiorida;
            Eimeriorina; Cryptosporidiidae; Cryptosporidium.
REFERENCE   1
  AUTHORS   Areeshi,M.Y., Hart,C.A. and Beeching,N.J.
  TITLE     Cryptosporidiosis in two regions of Saudi Arabia
  JOURNAL   Published Only in Database (2007)
REFERENCE   2  (bases 1 to 829)
  AUTHORS   Areeshi,M.Y.
  TITLE     Direct Submission
  JOURNAL   Submitted (29-NOV-2007) Contact:Mohammed Y Areeshi The University
            of Liverpool, Infection and Host Defence; 8th Floor Duncan
            Building, Daulby Street, Liverpool L693GA, United Kingdom
FEATURES             Location/Qualifiers
     source          1..829
                     /organism="Cryptosporidium hominis"
                     /mol_type="genomic DNA"
                     /isolate="Ar108"
                     /isolation_source="diarrhetic human child"
                     /db_xref="taxon:237895"
                     /country="Saudi Arabia"
                     /collection_date="05-Feb-2005"
                     /collected_by="Mohammed Areeshi"
     gene            <1..>829
                     /gene="HSP70"
     CDS             <1..>829
                     /gene="HSP70"
                     /codon_start=3
                     /product="70 kD heat shock protien"
                     /protein_id="BAF94160.1"
                     /db_xref="GI:161410629"
                     /translation="KQSIQMKLLLWCCCTSCYLNGEQSSAVQDLLLLDVAPLSLGLET
                     AGGVMTKLIERNTTIPAKKTQVFTTYADNQSGVLIQVYEGERAMTKDNHLLGKFHLDG
                     IPPAPRGVPQIEVTFDIDANGILNVSAVDKSTGKSSKITITNDKGRLSKDDIERMVND
                     AEKYKGEDEQNRLKIEAKNSLENYLYNMRNTIQEPKVKEKLSQSEIDEAEKKIKDALD
                     WLEHNQTAEKDEFEHQQKEIETHMNPLMMKIYSAEGGMPGGMPGGMPGGMPGGIEGG"
ORIGIN
        1 caaagcaatc aatccagatg aagctgttgc tatggtgctg ctgtacaagc tgctatctaa
       61 atggtgagca atcctctgcc gtacaggatc tcttattatt ggatgttgct ccattatcac
      121 tcggtttaga aactgctggt ggtgttatga ccaagcttat tgaacgtaat acaactatcc
      181 cagcaaagaa gacacaagtc ttcactactt atgctgataa ccagagcggt gtcttgatcc
      241 aagtttatga gggtgagaga gccatgacta aggataacca tctcctcgga aagttccatc
      301 ttgatggtat tccaccagca ccaagaggtg taccacaaat tgaagtcact tttgatattg
      361 atgctaatgg tatcttgaat gtatctgctg ttgataaaag cactggtaag agcagcaaga
      421 tcactattac taacgataag ggtagattat caaaggacga tattgaacgt atggttaatg
      481 atgctgagaa atacaagggt gaggatgagc aaaacagact caagattgag gctaagaact
      541 ctttggagaa ctacctctat aacatgagaa acaccatcca agaaccaaag gttaaggaaa
      601 agctttctca atctgaaatt gatgaggctg agaagaagat caaggatgct cttgactggc
      661 tcgagcacaa ccaaactgct gaaaaggacg agtttgaaca tcaacaaaag gaaattgaaa
      721 ctcatatgaa tccactcatg atgaagatct actctgctga gggtggtatg ccaggtggaa
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