Genetic Characterisation and Transmission Cycles of *Cryptosporidium* Species Isolated in New Zealand

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Abstract

Sixty-nine years separated the first observation of *Cryptosporidium* by Tyzzer in 1907 from the realisation in 1976 that this enteric protozoan parasite was pathogenic. It is the third major cause of diarrhoeal disease worldwide causing a self-limiting infection in immuno-competent humans and young vertebrates. As yet there is no antimicrobial agent that combats *Cryptosporidium* so the organism poses a life threatening risk to the immuno-compromised e.g., AIDS patients, patients on immuno-suppressive drugs, chemotherapy or congenital immune deficiencies. By 2000 AD 152 species of mammals had been reported as being infected with *Cryptosporidium* plus 57 reptilian species and many birds and fish.

The advent of AIDS stimulated research into *Cryptosporidium* resulting in the large amount of information now becoming available, however little is known about the genetic characteristics, distribution and transmission cycles of *Cryptosporidium* species that cause human disease in New Zealand. To address these questions 1613 animal faecal samples and 423 human faecal specimens containing *Cryptosporidium* oocysts were collected from throughout New Zealand and examined by the polymerase chain reaction - restriction fragment length polymorphism technique (PCR-RFLP). Indeterminant results were resolved by DNA sequence analysis of the small subunit ribosomal DNA (rDNA).

Only 2.8% of the animal faecal specimens contained oocysts with the vast majority of these being *C. parvum* bovine genotype from calves.

Two regions supplied the majority of human isolates, one rural and one urban. Overall *C. hominis* accounted for 47% of all human isolates with the remaining 53% being *C. parvum* bovine genotype. A difference however, was observed between the *Cryptosporidium* species from rural and urban isolates with *C. hominis* dominant in the urban region while *C. parvum* bovine genotype was prevalent in rural New Zealand. A shift in transmission cycles was detected between seasons with an anthroponotic cycle in autumn and a zoonotic cycle in spring. A novel *Cryptosporidium*, that on DNA sequence analysis showed a close relationship with *C. canis,* was detected in two unrelated children from different regions, illustrating the genetic diversity within this genus.

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As with any research of this nature and performed over a lengthy period there are many people to acknowledge, most of them inadequately. Those mentioned below played a major part in the completion of this project and hopefully persons not included in these acknowledgements will forgive me for the oversight.

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I well remember going to my first lecture in more years then I am willing to admit to, with Liz Keys. It was a DNA Technology lecture given by the inspiration educator Dr. Stowell, who started the lecture with "we will not have to go over this point as we covered it last year" – it was to become a recurring theme. I really wondered at times at what I was doing there, as must have the ever patient Dr. Stowell.

Collection of the human faecal specimens was carried out by a great many medical laboratory scientists from throughout New Zealand but special thanks go to Jan Bird, who at the time worked for MedLab Hamilton and Vicky McKnight from Medical Laboratory Wellington. Karen Cooper of Gribbles Veterinary Pathology diligently collected animal faecal specimens over many months, allowing me access to many potential *Cryptosporidium* hosts that I could not have otherwise examined. To collect positive faecal specimens and send them to the PRU takes a considerable effort when working in a busy diagnostic laboratory and I thank them all. Without Dr. Padraig Duignan I could not have searched through the faecal specimens from his collection of

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The perpetually busy Ina Te Wiata, who coincidentally was working with *Cryptosporidium* in the mid 1970s when the pathogenicity of the organism was being debated, helpfully proofread the thesis. Her many helpful comments were incorporated into this document.

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CHAPTER ONE: DETECTION, ISOLATION, VIABILITY AND GENETIC CHARACTERIZATION

1.1 *CRYPTOSPORIDIUM* BACKGROUND

1.1.1 History

Sixty-nine years separated the first observation of *Cryptosporidium* by Tyzzer in 1907 (138) from the realisation in 1976 that this protozoan was pathogenic (86, 100). *Cryptosporidium parvum* is the third major cause of diarrhoeal disease worldwide (34) causing a self-limiting infection in immuno-competent humans and young vertebrates. By 2000 AD 152 species of mammals had been reported as being infected with *Cryptosporidium* (40) plus 57 reptilian species (102) and many birds and fish.

As yet there is no antimicrobial agent that combats *Cryptosporidium* so it poses a life threatening risk to the immuno-compromised e.g., AIDS patients, patients on immuno-suppressive drugs, chemotherapy or congenital immune deficiencies. Up until the mid-1990s these patients often died from dehydration caused by a profuse and prolonged diarrhoea. Recently there have been large reductions in *Cryptosporidium* infection rates in AIDS patients who have received high activity anti-retroviral therapy (HAART) (106). HAART maintains the function of the immune system raising the CD4 lymphocyte count.

The traditional methods of oocyst flotation and acid fast staining, for the isolation and detection of *Cryptosporidium* from environmental samples plus human and animal faeces, have led to the under reporting of the prevalence of this parasite. This review looks at the methods currently available for the detection, isolation, viability assessment and genotyping of *Cryptosporidium*; appraising their advantages, disadvantages and suitability for the study of New Zealand isolates.

1.1.2 Biology

C. parvum is an obligate intracellular parasite with its entire life cycle taking place in the small intestine brush border of the same host (98). The haploid oocyst is the

infective environmentally resistant stage and contains four sporozoites. Excystation takes place in the gastrointestinal tract releasing the sporozoites to bind to receptors on the surface of the host's enterocytes (77). The enterocyte microvilli elongate and fuse around the sporozoite so that it is inside a parasitophorous vacuole that is extracytoplasmic. An electron dense organelle develops at the base of the parasitophorous vacuole and is believed to provide nutrient from the host cell (102). Here the sporozoites mature into merozoites that on release from the enclosing meront are free to infect other enterocytes. Following several cycles of asexual reproduction a small number of parasites commence a sexual cycle by differentiating into macro- and microgametes, which form zygotes after fusion and eventually oocysts (103). Mature oocysts may be either thick-walled which are free to pass from the host in faeces or thin-walled which excyst within the same host to amplify the infection (56).

1.1.3 Taxonomy

Cryptosporidium spp. are placed in the phylum Apicomplexa, order Eucoccidiorida, suborder Eimeriorina, family Cryptosporidiidae (98). Genera have been placed in this phylum based on their morphological features, unique life cycles and host specificity. It has been suggested that *Cryptosporidium's* unique position within enterocytes, lack of mitochondria, anticoccidial drug resistance, plant-type polyamine biosynthetic pathway, lack of introns in all genes with the exception of the β-tubulin gene, make the differences between *Cryptosporidium* and its closest coccidian neighbour enough to revise its phylogenetic position within the phylum Apicomplexa (161).

At the species level the taxonomy of *Cryptosporidium* is less accepted (122). Having comprised of 22 species at one time, based on host specificity, the number was revised in 2000 to 8 valid species (90) and currently numbers 12 or 13 valid species. *C. nasorum* is considered *nomen nudum* by some researchers (Table 1.) (118, 156). As a result of recent molecular studies the taxonomy of *Cryptosporidium* at this level needs reappraisal. The genetic variability of *Cryptosporidium* isolates from different geographical and host origins have been investigated by detecting polymorphisms in their iso-enzyme profiles (9), analysing selected and random genetic loci by polymerase chain reaction (PCR) techniques (5, 28, 37, 53, 57, 74, 84, 88, 90, 94, 96, 104, 110, 124, 132, 135, 143, 150, 160) and by direct sequencing (15, 18, 23, 29, 31, 35, 66, 89, 91,

112, 118, 119, 153, 155). These genetic, and in some cases biological differences, may see the naming of new host specific *Cryptosporidium* species.

Table 1. Described species of *Cryptosporidium*. Valid species of *Cryptosporidium* in bold and underlined.

Species	Host	Species	Host
C. andersoni	*Bos taurus* (ox)	*C. cuniculus*	*Oryctolagus cuniculus* (rabbit)
C. muris	*Mus musculus* (mouse)	***C. felis***	*Felis catis* (cat)
C. parvum	*Mus musculus* (mouse)	*C. rhesi*	*Macaca mulatta* (rheus monkey)
C. crotali	*Crotalus confluens* (rattlesnake)	***C. serpentis***	Crotalid, colubrid and boid snakes
C. vulpis	*Vulpes vulpes* (fox)	*C. garnhami*	*Homo sapiens* (man)
C. meleagridis	*Meleagris gallopavo* (turkey)	***C. nasorum***	*Naso literatus* (fish)
C. tyzzeri	*Gallus gallus* (chicken)	***C. baileyi***	*Gallus gallus* (chicken)
C. lampropeltis	*Lampropeltis calligaster* (king snake)	*C. curyi*	*Felis felis* (cat)
C. ameivae	*Ameiva ameiva* (lizard)	***C. saurophilium***	*Eumeces schneideri* (lizard)
C. ctenosauris	*Ctenosauris similis* (lizard)	***C. hominis***	*Homo sapiens* (man)
C. wrairi	*Cavia porcellus* (guinea pig)	***C. canis***	*Canis familiaris* (dog)
C. agni	*Ovis aries* (sheep)	***C. galli***	*Tetrao urogallus* (bird)
C. anserinum	*Anser anser* (goose)	***C. molnari***	*Sparus aurata* (fish)
C. bovis	*Bos taurus* (ox)		

That *Cryptosporidium* taxonomy is undergoing rapid changes is demonstrated by the recent name change of the organism that causes disease exclusively in humans from *C. parvum* genotype 1 or *C. parvum* human genotype to *C. hominis*. Investigations to genotype *Cryptosporidium* isolates from humans, regardless of the technique used have usually found only two species – *C. hominis* and *C. parvum* bovine genotype. *C. hominis* is found only in humans and is transmitted anthroponotically whereas *C. parvum* bovine genotype is responsible for animal and many human infections, being transmitted zoonotically. Less common species of *Cryptosporidium* have been reported in humans, often in association with an immunocompromised condition (53, 70, 90, 104, 108, 112, 146, 153, 157), which raises questions of taxonomy, epidemiology and

transmission cycles. To confuse matters more *C. parvum* bovine genotype is often termed *C. parvum* genotype 2 and *C. meleagridis* as *C. parvum* genotype 3 (83). With the ever-increasing number of novel *C. parvum* genotypes being recognised from environmental samples and vertebrate hosts, Thompson (2002) suggests that a numerical nomenclature should be discarded in favour of naming new species after their specific host (137).

1.2 *C. PARVUM* GENOME

That the scientific community is interested in *C. parvum* is attested to by the number of DNA sequences deposited in databases – about 40 in 1995 and by the year 2000 greater than 5200 (122). Three major genome sequencing projects (two in the USA and one in the UK) stimulated this culminating in the entire genomic DNA sequence of *C. parvum* being published in April 2004 (1). Two major benefits that will be derived from this project are the characterisation of polymorphic loci, allowing study of *C. parvum* populations at the genetic level and the identification of genes which may encode potential drug or vaccine targets (129).

1.2.1 Molecular genetics

The first attempt to analyse the *C. parvum* genome was carried out in 1988 using pulse field gradient electrophoresis (85). Results of this electrophoresis showed five chromosomal bands in the 1.4 to 3.3Mbp range. Subsequent work using contour-clamped homogenous electric field electrophoresis (13, 21, 69) showed the presence of eight chromosomes, labelled I to VIII, ranging in size from 0.94 to 1.44Mbp, for a total overall size of 9.4Mbp. One group (13) found a low molecular-weight molecule that may correspond to the 35Kbp circular, extra-chromosomal DNA found in other apicomplexan parasites. Other groups (122), however have been unable to reproduce these findings. This makes the *C. parvum* genome one of the smallest characterised of the protozoan parasites.

Two small, linear, extrachromosomal, virus-like, double-stranded RNAs (dsRNA) have been found in the cytoplasm of *C. hominis* and *C. parvum* bovine genotype sporozoites but not any other *Cryptosporidium* species or genotypes. One encodes an RNA polymerase and the other a protein with limited homology to mammalian mitogen-

activated c-Jun NH_2-terminal protein kinases (65). These novel molecules have been used as potential epidemiological markers in attempts to track outbreaks of cryptosporidiosis as they show sequence diversity in at least 17 sites (64, 152). Xiao *et al.* (2001) were able to find eight variants of the *C. parvum* bovine genotype and ten variants of *C. hominis* (152).

Nucleotide analysis of the *C. parvum* sequenced genes shows an elevated A/T content (approximately 65%) with a lack of introns (122). The β-tubulin gene is the exception, having a single intron of 85bps (20).

Comparison of *C. parvum* codon usage with other Apicomplexan members shows a greater similarity with the Haemosporida species rather than the Eimeriorina species to which it belongs (32). The lack of introns and the codon usage indicates that the Cryptosporidiidae have an evolutionary history unlike that of other members of the phylum Apicomplexa to which they are related (61).

1.2.2 Ribosomal gene organisation

The structure of rRNA genes is central to the phylogenetic classification and genotyping of many microbial organisms as they occur in all life forms with the exception of viruses and have a high level of conservation (134). In most eukaryotes, rRNA genes consist of three highly conserved regions; the 18S small sub-unit, the 5.8S sub-unit and the 28S large sub-unit. The variable internal transcribed spacer regions one and two (ITS-1 and ITS-2) divide these sub-units. Usually multiple copies of the gene exist per genome (*Toxoplasma* has 110) arranged in a head to tail tandem array on one or a few chromosomes. In some members of the phylum Apicomplexa (*Plasmodium, Theileria* and *Babesia* – Haemosporidia species) the rRNA gene structure remains the same but the copy number is very low (4 to 8, 2 and 3 respectively). These rDNA units are dispersed throughout the genome and the sequences are distinct (81). *C. parvum* follows this latter pattern having five rDNA unit copies per haploid genome dispersed over at least three chromosomes. At least two of the rDNA units are single unlinked copies on different chromosomes. Structurally there are two types of rDNA units, Type A and Type B, with the Type B unit having an extra 500bp in the ITS-1 region. Type A rDNA has four copies per haploid genome while the Type B unit has only the one (69). Thus

within the phylum Apicomplexa there are two opposite paradigms for rRNA gene organisation with the taxonomic position of *Cryptosporidium* being in conflict with its paradigm. This may be due to independent evolution by sexual linkages within the phylum or it may reflect an ancestral type so that *C. parvum* may have a closer relationship to the Haemospororina than the Eimeriorina (69).

Carreno *et al.* (1999) used rRNA sequences from a variety of Apicomplexan protozoa to construct phylogenetic trees by parsimony and maximum-likelihood methods. Organisms from the subclass Gregarinasina formed a sister clade to the *Cryptosporidium* species and this clade was separate from a clade containing the other genera of the Apicomplexa including the coccida and haemosporinids (29).

1.3 OOCYST DETECTION

Current laboratory methods for the detection of *C. parvum* oocysts from faeces and environmental samples include the modified Kinyoun's and Ziehl-Nelseen's acid-fast stains (45), the direct immunofluorescent monoclonal antibody stain (DFA), a reverse passive haemagglutination assay (39), enzyme immunoassays (EIA) (120), flow cytometry (43), and PCR techniques (148).

1.3.1 Acid-fast staining

The acid-fast stain allows many samples to be screened rapidly at low cost, however it has low sensitivity and specificity. Oocysts of other genera e.g., *Cyclospora* may cause confusion due to similar oocyst morphology. If the patient is excreting large numbers of oocysts, detection by this method should pose few problems. Variation in faecal consistency will affect detection efficiency with oocysts being more easily detected in watery diarrhoea specimens than in formed stools. The test is not suitable for oocyst detection in environmental samples due to its low sensitivity.

1.3.2 Immunological detection

DFA staining is sensitive and specific for *Cryptosporidium* but suffers from the high cost of reagents and the requirement for an epifluorescent microscope. Several manufacturers produce fluorescently labelled monoclonal antibody staining kits.

EIA techniques are also sensitive and specific but again there is a high cost for reagents and the necessity for a micro-plate reader. Recently EIA kits, which have *Cryptosporidium* antibodies impregnated on a membrane, have come on to the market. These capture and immobilise specific *Cryptosporidium* antigens from faecal specimens. While the kit performed well with *Cryptosporidium* in faeces, 98.3 % sensitive and 99.7% specificity from 60 positive samples, they are not suitable for environmental samples (46).

Flow cytometry's proponents claim that this is the best technique for detecting oocysts in environmental samples but the capital outlay for the flow cytometer and the ongoing maintenance costs are prohibitive for all but the largest water quality testing laboratories (141).

1.3.3 PCR techniques

PCR techniques are sensitive and specific, with one group detecting as few as one oocyst (145), plus they permit genotyping and viability studies. These techniques are non-standard, are high cost and require specialised training and equipment. Large numbers of samples can be batch processed reducing the cost and greatly improving the turn around time of oocyst detection. Morgan *et al.* (1998) compared acid-fast microscopy with PCR for detection of *C. parvum* oocysts in faeces (93). They examined 511 faecal specimens, detecting 29 positives by microscopy and 36 by PCR. Sensitivity and specificity by microscopy was 83.7% and 98.9% respectively while PCR sensitivity and specificity was 100%. An additional advantage of PCR was that it not only detected oocysts but it also identified genotypes.

Many workers are now combining the recovery efficiency of immunomagnetic separation (IMS) with the sensitivity and specificity of PCR to detect *Cryptosporidium* oocysts in environmental samples and faecal specimens. Substances that are inhibitory to PCR need to be removed first – humic acids in environmental samples and bile constituents in faeces. Johnson *et al.* (1995) used IMS to remove PCR inhibitors from environmental samples (62). This group considered IMS to be the best method for removal of PCR inhibitors. Previous to IMS they had used Chelex® 100 resin which also reduces inhibitors (142). Detection rates increased 100-fold when IMS was used

prior to PCR. Webster *et al.* (1996) reported detecting as few as five oocysts per ml of diluted faeces after IMS-PCR, representing "an increase of several orders of magnitude over conventional coprodiagnostic methods" available at the time (144). Deng *et al.* (1997) reported a 100-fold increase in detection of *C. parvum* oocysts from environmental samples when using IMS-PCR (38) and that it was also suitable for random amplified polymorphic DNA (RAPD) analysis, eliminating the long and laborious oocyst purification required (37). The QIAmp stool detection kit (Qiagen, Hilden, Germany), recently released, has been used for DNA extraction straight from the faecal specimens (119). While this bypasses the laborious oocyst purification step it would not be suitable for techniques requiring clean oocysts.

The TaqMan™ real-time PCR detection system (Perkin-Elmer) will detect and quantify *Cryptosporidium* simultaneously. Wiedenmann *et al.* (1998) are evaluating the technique for *Cryptosporidium* with initial results showing sensitivity similar to conventional PCR (148). Cost per assay will be high which will be offset by quick turnaround time of results. Tanriverdi *et al.* (2002) were able to discriminate between *C. hominis* and *C. parvum* bovine genotype using real-time PCR and melting curve analysis with the added advantage of speed (135). A nested real-time PCR technique was able to detect heterogeneous mixtures of *Cryptosporidium* species making it useful for environmental samples as well as faecal specimens (6)

For fluorescent *in situ* hybridisation (FISH) PCR, oocysts are made permeable, PCR reagents added and amplification takes place inside the oocyst with the amplified products unable to leave the oocyst. A fluorescently labelled oligonucleotide is hybridised to the amplicon and the product is detected inside the oocyst by epifluorescent microscopy. Prescott and Fricker (1999) are evaluating this method for RT-PCR of the 18S rRNA molecule in environmental samples. They report that the method shows potential for routine analysis of water and is able to genotype *Cryptosporidium* isolates (113).

Morgan and Thompson (1998) consider the combination of PCR and silicon chip technology to be the most exciting prospect on the horizon with the promise of speed and sensitivity (95). A microarray targeting single-nucleotide polymorphisms (SNPs) in the heat shock protein 70 gene (*hsp*70) was able to unambiguously distinguish *C. hominis* from *C. parvum* bovine genotype and had the potential to differentiate other

species on a single array (127). This powerful technique would lend itself to assessing water supplies as well as faecal specimens. The disadvantage of the technology is the requirement for specialised and expensive instrumentation.

1.4 OOCYST RECOVERY

Whatever method of oocyst recovery is used, oocysts need to be concentrated first and the background debris removed, although Morgan *et al.* (1997, 2003) perform PCR direct from the faeces (91, 119). Flotation techniques, flow cytometry or IMS will all remove background debris.

1.4.1 Flotation techniques

The two commonly used flotation techniques are sodium chloride (specific gravity 1.2) (144) and sucrose density gradient centrifugation (specific gravity 1.18) (SDGC) (139). Webster *et al.* (1996) found that DFA staining after salt flotation was less sensitive than DFA staining after sucrose flotation (144). This group suggested that the salt flotation method might have modified the epitopes on the oocyst wall, to which the antibody bound, in some way.

Many papers have mentioned the variable recovery rates obtained using SDGC and the "bible" of water quality testing laboratories (Standard Methods for the Examination of Waste and Wastewater) no longer lists the method in its latest edition claiming "lack of specificity, erratic efficiency, low precision and difficulty in determining viability" (33). Jakubowski *et al.* (1996) found in an inter-laboratory evaluations study that oocyst recoveries from water samples ranged from 0-140% (59) and our own laboratory found that recovery rates varied between 8 and 18%. A gram of oocyst containing faeces was concentrated in our laboratory by the formalin/ether method (4) and the oocysts recovered from the debris by SDGC. A total of 2.4×10^4 oocysts were recovered. Another gram of faeces from the same specimen was concentrated as previously and the oocysts recovered from the debris by IMS. This method gave a recovery of 2.4×10^6 oocysts.

We have compared the USEPA SDGC method with IMS by adding a series of doubling dilutions of oocysts, partially purified from bovine faeces, to 10ml aliquots of

laboratory tap water (17). Approximately 1 x 10^5 paramagnetic beads coated with monoclonal antibody to *Cryptosporidium* were added to each aliquot, mixed for one hour at room temperature and the bead oocyst complex recovered by using a magnet. The oocysts were washed, stained by DFA and enumerated. A second series was processed by the USEPA SDGC method. The results of the comparison show the greater recovery efficiency of IMS over the USEPA method (Table 2.)

An important disadvantage inherent in SDGC is the method's tendency to selectively recover non-viable and empty oocysts unsuitable for PCR (94).

Table 2. Recovery efficiency of the USEPA and IMS methods (17).

Oocyst conc./ml	USEPA method	USEPA efficiency (%)	IMS method	IMS efficiency (%)
4000	1154	29	3884	97
2000	720	36	1901	95
1000	336	34	958	96
500	132	26	445	89
250	63	25	231	92
125	31	25	109	87
64	7	11	53	83
32	3	9	28	88
16	0	0	14	88
8	0	0	6	75
4	0	0	3	75
2	0	0	0	0
0	0	0	0	0

1.4.2 Flow Cytometry

Cryptosporidium oocysts tend to adhere to particulate matter resulting in poor recoveries in turbid water samples, both for SDGC and IMS. Fricker and Crabb (1998) consider flow cytometry the most reliable method for separating oocysts from

background debris but this technique is only suitable for specialist laboratories for the reasons mentioned earlier (43).

1.4.3 Immunomagnetic separation

IMS uses paramagnetic beads that respond to a magnet but show no visible residual magnetism. Coated with streptavidin these beads bond to a biotin-conjugated antibody forming the basis of the technique. The success of the method lies in the streptavidin/biotin bond which has a strength approaching that of a covalent bond ($K_a = 10^{15}$/M) (12). Biotin conjugated monoclonal antibody to *C. parvum* is available from several companies. It is readily attached to the coated beads by mixing the two in phosphate buffered saline (PBS), pH 7.4, at room temperature for 0.5 hour with gentle mixing. After washing to remove non-bound antibody, the beads may be stored at 4°C in PBS.

Johnson *et al.* (1995) used IMS and a magnetic cell sorter (Perceptive Diagnostics, Inc.) to separate oocysts from environmental water samples (62). PCR sensitivity increased after background inhibitory substances had been removed. Polyclonal antibody was used in the oocyst capture but any cross-reaction problems with other organisms were countered by using *C. parvum* specific PCR primers.

Webster *et al.* (1996) isolated *C. parvum* oocysts by IMS from faecal specimens and detected their presence by PCR (144). They detected 5 oocysts/ml of diluted faeces and discussed the advantages of the removal of PCR inhibitors. By using SDGC the limit of detection was 100 oocysts/ml of diluted faeces.

Deng *et al.* (1997) differentiated between viable and non-viable oocysts recovered using IMS (38). Oocysts were sourced from infected calves, purified by SDGC and some rendered non-viable by treatment with 10% formalin, 10% ammonia, 90% ethanol or heated to 80°C for 1 minute. Both sets of oocysts were seeded into separate calf faecal specimens and re-isolated by IMS. After incubating the oocysts in excystation medium, viability was detected by PCR of nucleic acid released from the excysted *C. parvum* sporozoites. None of the non-viable oocysts excysted while 89% of the untreated oocysts excysted completely or partially.

Rochelle *et al.* (1999) compared two commercial IMS test kits (Crypto-Scan and Dynal) in a side-by-side evaluation (114). A series of recovery experiments were carried out in water samples seeded with varying amounts of *C. parvum* oocysts and varying in turbidity (range 370-11480 nephelometric turbidity units). Recoveries were performed according to the manufacturers instructions. The Dynal kit demonstrated higher recovery rates than the Crypto-Scan kit at all turbidity levels. The Crypto-Scan kit had a negative correlation between oocyst recovery and increased turbidity. At low oocyst levels (36 oocysts/10L) the Dynal kit recovered 35.5% more than the Crypto-Scan.

Determination of infectivity of recovered oocysts from environmental samples is important for public health purposes. One group demonstrated that by applying *C. parvum* antibody to oocysts it affected their *in vitro* infectivity (111). As IMS is an antibody based technique and could affect oocyst infectivity, Rochelle *et al.* (1999) inoculated HCT-8 and Caco-2 tissue culture cells with sporozoites from excysted IMS recovered oocysts (114). Tissue culture infection was determined by performing reverse transcriptase-PCR (RT-PCR) for the *C. parvum* specific heat shock protein 70 (hsp70) mRNA.

The USEPA has a new method for recovery of *C. parvum* oocysts from water – method 1623 (140). It is based on filtering 10L of water through a filter membrane contained in a capsular housing (Envirochek, Gelman). Oocysts are washed from the membrane in a small volume (250ml) of detergent solution and the oocysts recovered by IMS. This has become the next "standard" method for the collection and enumeration of *Cryptosporidium* oocysts from water for water quality laboratories.

1.5 VIABILITY

O'Donoghue (1999) suggests that the contamination of water with *Cryptosporidium* does not correlate well with disease occurrence (101). This would appear to be the case during the Sydney water contamination incident in 1998 where the water quality laboratory was detecting, on occasions, greater numbers of *Giardia* and *Cryptosporidium* than our laboratory detects in primary sludge. During this incident the background rates for giardiasis and cryptosporidiosis in the Sydney metropolitan area remained static (80). Frost *et al.* (2000) suggest that the number of oocysts may not have been as high as first thought, for their serological study comparing Sydney blood

donors to Melbourne blood donors using a *Cryptosporidium* antigen which indicates infection, showed no statistical significance in antibody response between the two populations (44). It must be remembered that there is a difference between viability and infectivity.

Fluorogenic vital dyes, *in vitro* excystation, tissue culture infectivity, animal infectivity and genetic analysis may be used to determine oocyst viability. All have deficiencies in their reliability.

1.5.1 Fluorogenic vital dyes

The fluorogenic vital dyes 6-diamidino-2-phenylindole (DAPI) and propridium iodide (PI) are commonly used to assess the viability of *Cryptosporidium* oocysts (26). Sporozoite nuclei that take up DAPI but fail to stain with PI are viable (DAPI+/ PI-). If PI is taken up as well the oocyst is non-viable (DAPI+/PI+). Exclusion of both dyes means the oocyst may or may not contain sporozoites (DAPI-/PI-). Jenkins *et al.* (1997) showed that dye permeability was a function of the oocyst wall rather than sporozoite metabolic activity (60).

Isolating enough oocysts for viability studies by vital dyes from environmental samples presents a problem as very few oocysts are normally recovered.

1.5.2 Tissue culture infectivity

These tests require PCR to detect viable *Cryptosporidium* at various stages of its life cycle. It is expensive, time consuming, labour intensive and some isolates may not infect cultured cells.

Meloni and Thompson (1996) infected MDCK, Caco-2, human fibroblasts, HT-29, a colon carcinoma cell line, and HCT-8 cells (87). They found the HCT-8 cells to best support the growth of *C. parvum.*

Deng and Cliver (1998) infected BS-C-1 cells, an African green monkey kidney cell line, with *C. parvum* bovine genotype and claimed it completed its whole life cycle in this tissue culture cell line (36). Whether *C. hominis* could infect this cell line is unknown.

All give peak growth at 48-72 hours and decline thereafter, unable to sustain intense infection by auto-infection.

1.5.3 Animal infectivity

This method suffers from the fact that each species and genotype of *C.* parvum is host adapted and then infection normally occurs only in young animals. For example, *C. hominis* does not infect mice (unless they are interferon-γ knockout mice) and insufficient numbers are usually isolated from environmental samples to constitute an infective dose.

1.5.4 Excystation and genetic analysis

PCR does not distinguish between DNA from viable and non-viable oocysts. By including an *in vitro* excystation step after oocyst isolation and purification (need oocysts free from contaminating free DNA), DNA from non-viable oocysts is eliminated. The assumption here is that if an oocyst does not excyst then it is non-viable (24). Deng *et al.* (1997) recovered oocysts by IMS and after washing obtained no false positive PCR results from free DNA (38). Neumann *et al.* (2000) working with *C. parvum* bovine genotype recovered non-excysted oocysts by flow cytometry, following an excystation step, with which they were able to infect neo-natal CD-1 mice (99). As a complicating factor excysted sporozoites can appear viable and be non-infectious (41). The limiting factor here may be the sensitivity of the excystation conditions.

1.5.5 Reverse transcription-PCR (RT-PCR) of messenger RNA (mRNA)

Another approach is to detect mRNA by RT-PCR on the basis that live organisms carry out transcription to form mRNA. Detection of viability by RT-PCR of a target mRNA does not depend on *in vitro* excystation but the mRNA must have a short half-life and be undetectable in non-viable organisms. Weidenmann *et al.* (1998) have suggested using hsp70 as a target mRNA as it meets the above conditions. In viable cells, heat shock proteins are important in the correct folding of polypeptides during their synthesis so they are always present at a low level. When a cell dies the instability of hsp70 mRNA at ambient temperature coupled with its short half-life means that it is not

detected in non-viable oocysts. Oocysts are recovered by IMS, washed, and stressed by heating. Hsp70 mRNA transcripts increase 1000 to 10000-fold under these conditions (63, 126) but quickly drop to levels 2-4 times as high as before the temperature increase. A cautionary note comes from Gobet and Toze (2001) who, working with inactivated heat shocked oocysts found that DNase-treated lysates were positive for hsp-70 mRNA and negative for lysates where the mRNA was recovered by IMS (50). More work continues presently in our laboratory on this technique.

1.6 MOLECULAR CHARACTERISATION OF *CRYPTOSPORIDIUM*

As there is no standard reference strain of *Cryptosporidium*, due to long term storage of viable oocysts being unavailable, comparative studies of isolates from different hosts and geographical regions is difficult. Identifying *Cryptosporidium* isolates to the species and genotype level is also hindered by the lack of a standard reference strain. Over the years there has been growing evidence based on genetic variation and biological differences that *C. parvum* was not a uniform species. There were two distinct genotypes which infected humans (*C. parvum* genotype 1 and genotype 2), one of which can also infect humans and animals. Morgan *et al.* (1999) suggested that these be considered two different species (90). These genetic differences have become apparent through analysis of polymorphisms detected by RAPDs (28, 37, 49, 92, 96), PCR-RFLP and/or sequence analysis (10, 15, 18, 23, 28, 29, 31, 57, 66, 74, 84, 88, 89, 91, 107, 110, 112, 124, 134, 146, 150, 153, 155). At the Freemantle, Australia, 2001 conference "*Cryptosporidium*: from Molecules to Disease" it was generally agreed that these two genotypes of *C. parvum* would become two species. Further to this conference Morgan-Ryan *et al.* (2002) formally proposed the case for *C. parvum* genotype 1 to become the new species *C. hominis* (97). Since the publication of their paper *C. parvum* genotype 1 has been termed *C. hominis*.

1.6.1 Nucleic acid extraction

The wall of the *Cryptosporidium* oocyst is extremely robust with the nucleic acids resisting release by boiling. Methods of releasing nucleic acids include treatment with Proteinase K for up to 48 hours (10), up to 10 cycles of rapid freeze and thaw (68), sonication (121) and excystation with the liberated sporozoites disrupted by boiling

(37). The latter method only works if the oocysts are viable and of the others Sluter *et al.* (1997) found three cycles of freeze/thaw gave the best result (121).

1.6.2 Random amplified polymorphic DNA analysis

This is a technique based on PCR using primers of arbitrary sequence. RAPDs require small quantities of DNA, no sequence knowledge is necessary and it is technically easy to perform. The disadvantage of the method is the long and laborious oocyst purification needed to remove contaminating free DNA. Deng and Cliver (1998) found IMS gave oocysts pure enough to give reproducible results (37). All groups confirmed the difference between human and animal species by RAPDs (28, 37, 49, 95, 96). Rochelle *et al.* (1999) reported RAPD fingerprint patterns generated in their laboratory had little resemblance to those generated in another laboratory, even though they used the same primers and DNA template (54, 116). They put this difference down to using a different thermal cycler and do not recommend the technique for routine molecular fingerprinting.

To overcome the problems with RAPDs most genetic characterisation studies have used *Cryptosporidium* specific PCR primers.

1.6.3 Sequencing and/or PCR-restriction fragment length polymorphism analysis

The loci analysed by sequencing and/or PCR-RFLP of *Cryptosporidium* include the various subunits of the rRNA gene (15, 23, 31, 69, 74, 88, 89, 91, 112, 134, 150, 153, 155), the *Cryptosporidium* outer wall protein gene (84, 107), the beta-tubulin gene (115, 146), the thrombospondin-related adhesive proteins C1 (124, 150) and C2 (153), the acetyl-CoA synthetase gene (89), the translation initiation factor eIF-4A (123), the ribonucleotide reductase gene, RNR R1 (3, 147), the dihydrofolate reductase-thymidylate synthase gene, DHFR (48, 105), the actin gene (156), the 60-kDa glycoprotein gene, GP60, also called *Cpgp*15/45 (5, 73, 149), a poly threonine repeat unit, poly-T (27, 159) and the 70-kDA heat shock protein gene, hsp70 (50, 51, 127, 131, 156). All of these studies confirmed the genetic distinctions of the human and animal isolates but they have also shown very little variation within the two genotypes with the exception of GP60.

Widmer *et al.* (1998) found polymorphisms within *C. hominis* and evidence of recombination between human and animal genotypes in the β-tubulin intron (146), however there is no support for these results (26). This group was performing PCR directly from faeces so the possibility that contaminating DNA was amplified cannot be discounted.

Morgan *et al.* (1999) looked at sequence heterogeneity between isolates of *Cryptosporidium* from a variety of hosts in the ITS-1 and ITS-2 rDNA. *Cryptosporidium* isolates from six mammalian hosts (cat, cattle, human, koala, mouse, and pig) were sequenced over the length of the ITS-1 and ITS-2 regions of Type A rDNA. Primers were designed from sequences in the conserved 18S and 28S rDNA sub-units. They found substantial differences between the isolates from different hosts but minimal variation between isolates from the same host species (88). The length of the ITS-1 region ranged from 363bp for the cat to 618bp for the koala and the A/T richness ranged from 82% for pig isolates to 91% for the human (Table 3.). PCR-RFLP analysis of the ITS-1 region from the six hosts, using the restriction endonuclease *Dra*1, gave six specific and reproducible fingerprint profiles corresponding to the six different hosts. Fragment sizes were as predicted from sequence data (Table 4.). This evidence makes a taxonomic revision of the genus *Cryptosporidium* look necessary, as there appears to be a variety of distinct species within *C. parvum*.

Table 3. Length and percentage AT richness of the ITS-1 and ITS-2 rDNA regions of *Cryptosporidium* isolates from different hosts (88).

Host	Length of ITS-1 (bp)	ITS-1% AT rich	Length of ITS-2 (bp)	ITS-2 % AT rich
Cattle	496	88	508	88
Cat	363	83	585	80
Human	523	91	566	88
Koala	618	84	497	86
Mouse	534	88	518	88
Pig	607	82	479	79

Table 4. Amplicon lengths and *Dra*1 restriction fragment sizes for the ITS-1 region of *Cryptosporidium*i isolates from different hosts (88).

Host	Size of PCR product (bp)	No. of *Dra*1 sites (TTT/AAA)	Expected restriction fragment sizes (bp)
Cattle	847	1	598, 249
Cat	714	1	617, 97
Human	884	4	348, 174, 132, 126, 104
Koala	970	1	721, 249
Mouse	884	1	625, 259
Pig	958	1	670, 288

1.6.4 Microsatellites

Due to the conserved nature of the DNA sequence targets used to detect *Cryptosporidium* species a method is needed for higher resolution of sub-genotypes within a species. This would have implications for tracking sources of contamination and outbreaks. The various microsatellite, SNPs, viral-like dsRNA, and microarray methods are a response to this problem.

Microsatellite DNA sequences normally consist of up to 50 tandem repeats of dinucleotide or trinucleotide motifs. Usually they are highly polymorphic in length at a given locus due to uneven crossover or slippage of DNA polymerase at replication. There are great numbers of these repeats throughout the genome of most eukaryotes (2, 122).

Caccio *et al.* (2000) looked at the GAG trinucleotide at a specific locus (GenBank accession number G35358) and using this technique they could sub-divide *C. hominis* into two sub-genotypes and *C. parvum* bovine genotype into four sub-genotypes (18).

Feng *et al.* (2000) identified 14 microsatellites in non-coding regions of the genome made up of mono-, di-, and trinucleotide repeats of A, AT, and AAT. These loci showed

significant length polymorphism. Up to seven alleles with a difference in length ranging between 1 and 27 bp were found at a single locus (42).

A study by Mallon *et al.* (2003), of 180 isolates collected from a discrete geographic area over a set time period, to examine population structures and the role of genetic exchange, looked at seven polymorphic microsatellites. They concluded that a higher resolution method might be required for *C. hominis* and *C. parvum* populations which may not be stable in time and place. It was emphasised that populations over larger geographic distances would need to be studied (78).

1.6.5 Single nucleotide polymorphisms

The gp15/45 single copy gene is the most polymorphic *Cryptosporidium* gene examined to date. It has five to six allelic classes defined by numerous SNPs and single-amino-acid polymorphisms (SAAPs). These alleles are characterized by additional intra-allelic sequence variation (5, 73, 109, 128, 130, 149).

An oligonucleotide microarray specific for *Cryptosporidium* species was designed by Straub *et al.* (2002) to target seven SNPs within the hsp70 gene (127). More work is required on the optimization of the microarray but it does provide a powerful tool for tracing contamination of environmental and treated water. After extensive study of the hsp70 gene DNA sequence by Sulaiman *et al.* (2000, 2001) they conclude that the gp15/45 gene gives higher resolution gene fingerprinting (130, 131).

1.6.6 Viral-like double-stranded RNA

Extrachromosomal dsRNA genetic elements are widespread in the pathogenic protozoa with *Cryptosporidium* being no exception. Khramstov *et al.* (1997) discovered two small, linear extrachromosomal dsRNA molecules in the cytoplasm of the sporozoites (65). They have been found only in *C. hominis* and *C. parvum* bovine genotype. By sequencing the complementary DNA (cDNA) product from RT-PCR this group was able to divide *C. hominis* into five variants and *C. parvum* bovine genotype into nine variants (64). Using the same method Xiao *et al.* (2001) found ten variants within *C. hominis* and divided *C. parvum* bovine genotype into eight variants (152).

By using a heteroduplex mobility assay Leoni *et al.* (2003) identified eight *C. hominis* and 12 *C. parvum* bovine genotype variants (75, 76).

1.7 OBJECTIVES AND APPROACHES

Cryptosporidium is now recognised worldwide as an important gastro-intestinal parasite infecting the host by the faecal-oral route. Faecal contamination of drinking water, swimming water, recreational water, raw and insufficiently pasteurised milk, cider and berries have all caused human infections and epidemics (30). The 1983 epidemic due to *Cryptosporidium* contamination of Milwaukee's drinking water supply affected greater than 400,000 people, including 108 deaths. It was at first thought to be caused by animal contamination from farm run-off (82) but genotyping indicated that the *C. hominis* was the causative organism (110).

This project genetically characterises the human and animal *Cryptosporidium* species present in New Zealand by analysing sequence polymorphisms in the ribosomal DNA (rDNA). Spatial and temporal features of the species were investigated as public health surveillance data shows that there are two seasonal peaks per year of human cryptosporidiosis (11). To date no investigations have looked at these peaks with regard to the *Cryptosporidium* species or to the geographic location of the species.

Initially the internal transcribed spacer 1 region (ITS-1) of the rDNA was investigated, for its suitability to differentiate the species by PCR-restriction fragment length polymorphism (PCR-RFLP) but subsequently the more conserved small sub-unit 18S rDNA was used. Internationally this is the area of the genome that is used for taxonomic purposes. The 18S rDNA of new isolates from animals, other than humans and cattle, was sequenced and aligned with known sequences.

Other loci examined included the β-tubulin gene, the poly-threonine repeat unit, the *Cryptosporidium* outer wall protein (COWP) gene and the ribonuclease reductase R1 (RNR R1) gene.

The viral-like dsRNA sequence will be analysed for its ability to sub-genotype isolates of *C. hominis* and *C. parvum* bovine genotype. This may be useful in tracing the source of *Cryptosporidium* outbreaks and in investigating contaminated water and food.

The aim of this project is to utilise the most suitable method for *Cryptosporidium* oocyst detection, isolation, and oocyst viability determination. Long-term objectives of this study are to examine transmission cycles and genetically fingerprint isolates from outbreaks and water contamination.

The significance of this project will be in the epidemiological and transmission cycle information gained from New Zealand humans and animals. This knowledge will hopefully contribute towards the betterment of New Zealand public health.

CHAPTER TWO: MATERIALS AND METHODS

2.1 COLLECTION OF *CRYPTOSPORIDIUM* OOCYSTS

2.1.1 Oocyst collection from dairy farms

An outside agency contracted the Massey University Protozoa Research Unit (PRU) to examine faecal samples from 65 dairy herds in the Waikato for the presence of *Cryptosporidium* oocysts and *Giardia intestinalis* cysts. Approximately 25 grams of faecal material was collected from the rectum of cows at milking and sent to the PRU within 24 hours. Between August 2000 and October 2000 samples from 170 cows and 165 calves were collected and examined. A second sampling was carried out between January 2001 and May 2001 on 174 cows and 129 calves. Other faecal specimens collected from the Waikato dairy farms but not of bovine origin included samples from 30 ducks, 13 hares, 22 rabbits and 50 possums.

Faecal specimens collected by the same manner were obtained from 170 cows and 14 calves from the Massey University dairy farms and 64 calves on a PRU member's family farm in the Tararua district.

2.1.2 Oocyst collection from medical diagnostic laboratories

Human faecal specimens containing *Cryptosporidium* oocysts were received from 13 medical diagnostic laboratories over a three year period, with the majority coming from MedLab Hamilton and Medical Laboratory Wellington (Table 5.). The last positive specimen was received in September 2003.

All patient demographics were removed from the faecal containers due to the legal requirements of the Privacy Act 1993, with the exception of the date collected and the referring laboratory's specimen number.

2.1.3 Oocyst collection from veterinary diagnostic laboratories

Over the period of the study, samples suspected of having a *Cryptosporidium* infection were received from a number of veterinary and animal diagnostic laboratories (Table 6.).

Table 5. Medical diagnostic laboratories participating in a nationwide survey of *Cryptosporidium* species infecting humans in New Zealand.

Medical Laboratories	Number of positive specimens
Northland Pathology	6
MedLab Hamilton	113
MedLab Bay of Plenty	33
Diagnostic Rotorua Laboratory	2
MedLab Hawkes Bay	30
Southern Communities Laboratory (Hastings)	6
MedLab Central (Palmerston North)	6
Masterton Hospital	1
Valley Diagnostic Laboratories (Hutt Valley)	25
Hutt Valley Pathology	1
Medical Laboratory Wellington	122
Healthlab Otago	1
MedLab Southland	35

The samples from Massey University's Institute of Veterinary, Animal and Biomedical Sciences (IVABS) included samples collected at the small animal clinic, 20 faecal samples from a number of marine mammals obtained at autopsy and preserved in formalin, plus faecal samples from 44 seagulls.

Table 6. Veterinary and animal diagnostic laboratories which referred specimens for *Cryptosporidium* species detection.

Veterinary laboratories	Number of referred samples
AgriQuality*	368
IVABS	94
Gribbles Veterinary Pathology	88
Small Animal Production Unit	19
ESR	63

*AgriQuality at Auckland, Ruakura, Palmerston North, Christchurch

2.2 DETECTION OF *CRYPTOSPORIDIUM* OOCYSTS

2.2.1 Modified Ziehl-Nelseen's acid-fast stain

Henriksen and Pohlenz's (1981) modification of the Ziehl-Neelsen acid-fast stain was used to detect *Cryptosporidium* oocysts from animal faecal samples, substituting Löffler's methylene blue for the original 5% malachite green counterstain (55).

Materials

- Ziehl-Nelseen's carbol fuchsin
- Sulphuric acid, 4% solution
- Löffler's methylene blue
- Formalin, 10%
- Microscope slide coverslips (22 x 60 mm), #1 thickness

Ziehl-Nelseen's carbol fuchsin

Basic fuchsin	10 g
Absolute ethanol	100 ml
Phenol solution (5% in distilled water) to	1000 ml

Dissolve the fuchsin in the ethanol and add to the phenol solution.

Sulphuric acid, 4% solution

Sulphuric acid, concentrated (~1.835 g per ml)	40 ml
Distilled water to	1000 ml

Place approximately 900 ml of distilled water in a volumetric flask and pour the concentrated acid slowly down the side of the flask into the water. Top up to 1000 ml and mix gently.

Löffler's methylene blue

Methylene blue, saturated solution in ethanol	300 ml
Potassium hydroxide, 0.01% w/v	1000 ml

Formalin, 10%

Commercial formalin (40% formaldehyde w/v in water)	100 ml
Distilled water to	1000 ml

Method

An approximate 20% suspension of faeces in 10% formalin was allowed to stand for 5 min to inactivate microbes. A drop of faecal suspension (~50 µl) was spread on to a glass slide over approximately 2.0 cm^2 and allowed to air dry before fixing for ten minutes in absolute ethanol. Carbol fuchsin solution was used to flood the slides at room temperature for one hour. The slides were washed in water and then the film decolourised with 4% sulphuric acid for between 15 s and 1 min, depending on film thickness. The slides were again washed quickly in water and counterstained for 1 min with Löffler's methylene blue solution. After a final wash the slides were air dried and examined at 250X and 500X magnification.

2.2.2 Direct immunofluorescent antibody stain

Materials

- Formalin, 10% (see Section 2.2.1.)
- Merifluor® *Cryptosporidium/Giardia* direct immunofluorescent detection kit, (Meridian Bioscience, Inc., Cincinnati, Ohio.).
- Phosphate buffered saline (PBS) 10X stock (pH 7.4)

- PBS + 0.2 % polyoxyethylene sorbitan monolaurate (Tween 20)
- Evans blue solution, (bioMerieux® sa, Marcy-Etoile, France)
- Fluoprep, mounting fluid, (bioMerieux® sa, Marcy-Etoile, France)
- Microscope slide coverslips (22 x 60 mm), #1 thickness.

Phosphate buffered saline, 10X stock solution (pH 7.4)

Sodium chloride	80 g
Potassium chloride	2 g
Potassium dihydrogen phosphate	2 g
Disodium hydrogen phosphate; dodecahydrate	29 g
Distilled water to	1000 ml
Adjust pH to 7.4	

PBS Tween 20

Dilute 1 volume of PBS 10X stock solution with 9 volumes of distilled water, check pH is 7.4 and add 2 ml of Tween 20.

Method

The manufacturer's instructions were followed with minor variations for the sake of economy. Kit contents used included fluorescein-isothiocyanate (FITC) labelled anti-*Cryptosporidium*/anti-*Giardia* monoclonal antibodies in solution, 20X wash buffer solution, *Giardia* and *Cryptosporidium* (oo)cyst positive and negative formalin treated faecal suspensions, and printed slides with treated wells (three per slide).

An approximate 20% suspension of faeces in 10% formalin was allowed to stand for 5 min to inactivate microbes. One drop of faecal suspension was spread over the entire well of a treated slide, allowed to air dry and fixed with absolute ethanol for 10 min. Positive and negative faecal controls were tested with each batch of specimens examined. The FITC labelled monoclonal antibody was diluted 1 in 10 with PBS and one drop spread over each well. Slides were incubated in a humidified chamber, in the dark for 30 min. Two Coplin jars were filled with 50 ml of 1X wash buffer (can substitute PBS Tween 20) and two drops of Evans blue counterstain mixed in the second Coplin jar. The slides were briefly placed into the 1X wash buffer in the first Coplin jar and immediately transferred to the second Coplin jar with the 1X wash buffer

plus Evans blue. After 1 min in the counterstain the slides were removed and the excess wash buffer drained off. One drop of Fluoprep was a placed on each well and a coverslip applied. The slides were not allowed to dry at any stage before mounting. Examination of the slides was performed under ultraviolet light with an Olympus BX60 epifluorescent microscope fitted with a filter system suitable for FITC; excitation wavelength 490 nm and emission wavelength 520 nm. This microscope was also equipped for Normaski differential interference contrast (DIC) microscopy, phase contrast microscopy and was mounted with a DP10 digital camera system. Each well was scanned at 250X magnification and suspect oocysts confirmed at 500X magnification. Positive oocysts stained a bright apple green against a reddish orange background and were 4.5 – 5.0 μm in diameter. A suture line was visible on a small percentage of oocysts.

2.2.3 Differential interference contrast microscopy

Materials

- Olympus BX60 microscope with a DIC system
- Oocyst suspension (from Section 2.3.2.)

Method

This method was used to check the condition of the oocysts isolated in Section 2.3.2.

A 15 μl drop of the oocyst suspension was put on a clean microscope slide and a 22 X 22 mm coverslip carefully placed on top. The suspension was scanned initially at 250X magnification and oocysts examined at 500X. Healthy viable oocysts had a three dimensional refractile appearance and contained sporozoites that filled the interior of the oocyst. Nonviable oocysts contained a small discrete mass in the centre of the oocyst that appeared to have pulled away from the oocysts wall, leaving the majority of the oocyst empty. Excysted oocysts lacked any contents and appeared non-refractive with the oocyst wall split at one point.

2.3 ISOLATION OF *CRYPTOSPORIDIUM* OOCYSTS

2.3.1 Oocyst concentration

Materials

- Formalin, 10% (see Section 2.2.1.)
- Diethyl ether

Method

All human samples were processed by this method as they had been previously found to contain oocysts by the referring medical diagnostic laboratories. Only those animal faecal samples that contained oocysts, as shown by either the modified Ziehl-Nelseen acid-fast stain or the direct immunofluorescent antibody stain, were processed by this technique.

Using two applicator sticks approximately 2 g of faeces were thoroughly mixed and suspended in 7 ml of 10% formalin in a disposable, capped 15 ml conical centrifuge tube. Large particulate material was removed by passing the faecal suspension through a metal sieve (aperture size 425 μm) into a new 15 ml tube. Working in a fume cupboard, 3 ml of diethyl ether was added to the faecal suspension and vigorously mixed for 30 s. This mixture was centrifuged for 1 min at 750X g. Faecal fat, which formed a plug at the top of the tube, was rimmed with an applicator stick and poured off along with the fluid phase containing faecal debris. *Cryptosporidium* oocysts were concentrated at the bottom of the tube amongst any heavy faecal debris present in the original specimen.

2.3.2 Immunomagnetic separation

Materials

- Dynabeads® GC-Combo immunomagnetic bead separation kit, (Dynal Biotech ASA, Oslo, Norway)
- 0.1M Hydrochloric acid
- 1.0M Sodium hydroxide

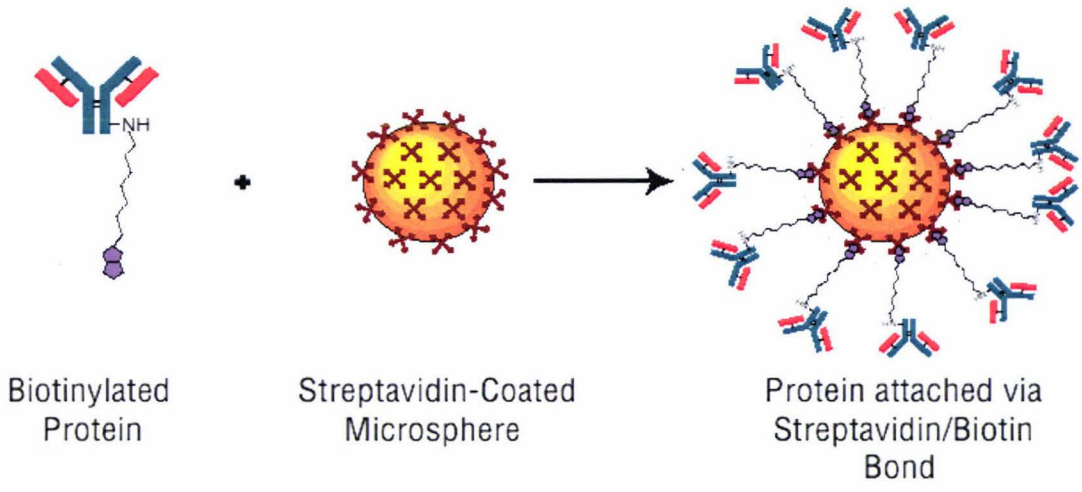


Figure 1. Coupling of biotinylated *Cryptosporidium* monoclonal antibody to streptavidin coated paramagnetic beads (12).

A process whereby a magnetite, styrene, divinylbenzene mixture is dispersed in water and the suspension polymerised to trap the magnetite in the polymer matrix produces the uniform, paramagnetic, microscopic beads. The magnetite is dispersed throughout the bead, which is finally coated with streptavidin. *Cryptosporidium* antibody, conjugated to biotin, is attached to the streptavidin and the beads suspended in PBS, pH 7.4 plus 0.1% bovine serum albumin (BSA) (Figure 1).

Method

The manufacturer's instructions were followed with minor variations. Kit contents included 10X SL™ buffer A, 10X SL™ buffer B and 1ml of anti-*Cryptosporidium* Dynabeads®.

Oocyst concentrate (from Section 2.3.1.) was made up to a total volume of 800 µl with distilled water in a 1.5 ml micro-centrifuge tube. Added to the suspension were 100 µl of 10X SL™ buffer A, 100 µl of 10X SL™ buffer B and 25 µl of fully resuspended anti-*Cryptosporidium* Dynabeads®. A LABQUAKE® shaker/rotisserie (Barnstead/Thermolyne, Dubuque, IO, USA) was used to gently rotate the mixture for one hour at 8 RPM. Separation of the now bound oocyst-Dynabeads® from the faecal debris was achieved by way of an in house built micro-centrifuge holder containing a magnet. The heavy faecal debris was discarded before removing the micro-centrifuge

tube from the holder and the oocyst-bead complex resuspended in 1 ml of 1X SL™ buffer A (10X buffer A diluted 1:10 with distilled water). Again the oocyst–bead complex was placed in the magnetic holder to immobilise the complex and the wash buffer discarded. This wash procedure was performed once more to ensure the oocysts were free from any debris. Oocysts were separated from the beads by the addition of 50 µl of 0.1 M HCl for 10 min with 10 s of vortexing at the start and end of the incubation period. The fluid contents from the micro-centrifuge tube, containing the clean oocysts, were transferred to a 650 µl microtube and the pH returned to neutral by the addition of 5 µl of 1M NaOH. Presence of healthy oocysts was checked by DIC.

2.4 EXTRACTION OF NUCLEIC ACID FROM *CRYPTOSPORIDIUM* OOCYSTS

2.4.1 Nucleic acid extraction

Materials

- TE buffer + 1% Nonidet P40
- Chelex® 100 resin suspension
- Liquid nitrogen
- Waterbath at 96°C

TE buffer (Tris-EDTA) + 1% Nonidet P40

1M Tris-HCl (pH 7.5)	10 ml
0.2M EDTA disodium salt (pH 7.2)	5 ml
Nonidet P40 (BDH, Poole, England)	10 ml
Distilled water to	1000 ml

1M Tris-HCl buffer (pH 7.5)

Trizma base (Sigma, St. Louis, MO)	12.11 g
HCl to pH 7.5	
Distilled water to	100 ml

Chelex® 100 resin 20% suspension

Chelex® 100 resin, 100 – 200 mesh, sodium form	
(BIO-RAD, Hercules, CA)	20 g
Distilled water to	100 ml

Method

To 55 µl of oocysts previously isolated (Section 3.2.2.) in a 650 µl microtube, 75 µl of TE buffer containing 1% Nonidet P40 and 20 µl of a 20% of Chelex® 100 resin were added. A #22 gauge syringe needle was used to puncture the microtube caps, preventing the rapid build up of pressure during the freeze and thaw cycles. A total of five freeze/thaw cycles was used to extract the nucleic acids. Oocysts were subjected to liquid nitrogen for 2 min before being rapidly transferred to a 96°C waterbath for 2 min. The microtubes were placed in a polystyrene float so that the bottom two thirds of the microtubes were immersed in either the liquid nitrogen or the 96°C. Cell debris and Chelex® 100 resin were deposited by centrifugation at ~17,900X g for 1 min. The supernatant containing the nucleic acids was collected into a new microtube, making sure that no PCR inhibiting Chelex® 100 was transferred and stored at 4°C until required.

2.5 PCR AMPLIFICATION OF *CRYPTOSPORIDIUM* LOCI

2.5.1 PCR amplification of a generic *Cryptosporidium* DNA locus

Materials

- *Cryptosporidium* oocyst nucleic acid extract
- PCR reagents
  - PCR buffer 10X - 200 mM Tris-HCl (pH 8.0), 500 mM KCl (Invitrogen™, Carlsbad, CA)
  - Magnesium Chloride, 50 mM (Invitrogen™, Carlsbad, CA)
  - *Taq* DNA polymerase, 5 units/µl (Invitrogen™, Carlsbad, CA)
  - Deoxynucleoside triphosphate set, 100 mM concentrations of dATP, dCTP, dGTP and dTTP (Roche, Mannheim, Germany). Diluted to 2 mM for use.

- Appropriate primers (Invitrogen™, Carlsbad, CA), stock solution of 1mM; diluted to a working strength of 20 µM
- Autoclaved distilled water

- Ultra pure DNA grade agarose (BIO-RAD, Hercules, CA)
- 1 Kb Plus DNA ladder - 1 µg/µl in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl and 1 mM EDTA (Invitrogen™, Carlsbad, CA)
- E buffer 10X
- Ethidium bromide (BDH, Poole, England)
- SDS loading dye 10X

E buffer 10X

Tris (Invitrogen™, Carlsbad, CA)	48.44 g
EDTA disodium salt	3.72 g
Sodium acetate, anhydrous	4.1 g
Distilled water to	1000 ml

Adjust pH to 7.8 with glacial acetic acid before diluting 1:10 with distilled water for use.

SDS loading dye 10X

Sucrose	8 g
EDTA disodium salt	74 mg
Sodium dodecyl sulphate (SDS)	100mg
Bromophenol blue (BDH, Poole, England)	10mg
Distilled water to	20 ml

Aliquot into 5 ml Bijoux bottles and store in the dark at room temperature.

Ethidium bromide solution

Ethidium bromide	25 µg
E buffer 1X	50 ml

Stored in a bottle sealed against light

As ethidium bromide is mutagenic, appropriate care must be taken when handling and disposing of this chemical.

Method

All animal and human faecal specimens positive for *Cryptosporidium* oocysts were amplified using one of the six loci documented in Section 2.5.2., before either restriction enzyme digestion and analysis of the restriction fragment lengths or direct sequencing of the PCR product.

Sterile technique was used at all times. All liquid manipulations were performed in a Class II biological safety cabinet with a set of PCR dedicated pipettes (Finnpipette, Helsinki, Finland), fitted with sterile barrier filter tips (Molecular BioProducts, San Diego, CA). Sterile latex gloves were worn and changed often during the procedure.

The PCR reactions were performed in 0.2 ml thin-walled thermo-tubes with flat caps (Abgene®, Surrey, England). All reagents and reaction tubes were kept on ice to lessen the chance of non-specific priming. To minimise pipetting and lessen errors a cocktail of the appropriate reagents was prepared when a batch of multiple samples was processed. This cocktail of reagents was mixed to give a total volume of 19 µl. Each reaction, regardless of the loci to be amplified contained as a final concentration;

PCR buffer 1X	2 µl
dNTP, 250 µM of each	2.5 µl
$MgCl_2$, 1.5 mM	0.6 µl
appropriate primers, 1 µM	1.0 µl of each
Taq polymerase, (5 units/µl)	0.5 µl
distilled water, autoclaved	11.4 µl

The *Cryptosporidium* DNA template was added last as a 1 µl volume to give a total reaction volume of 20 µl, after which the thermo-tubes' contents were mixed and briefly centrifuged at approximately 5,000X g to deposit the reactants at the bottom of the tubes. A negative control in which water was substituted for template DNA was processed with each PCR batch. As all the specimens contained *Cryptosporidium* oocysts a positive control was not considered necessary. Amplification of the DNA template was performed in a Perkin Elmer Gene Amp® PCR System 9700 thermal

cycler (Applied Biosystems, Foster City, CA) using version 3.01 software. The PCR cycling conditions for the individual specific loci are described in Section 2.5.2. At the end of the cycle programme the reaction temperature was held at 4°C until the amplified products were removed for examination by gel electrophoresis.

Ultra pure DNA grade agarose at a final concentration of 1.6% was dissolved in E buffer 1X and 5 mm gel slabs prepared in either a Horizon 58 horizontal gel box (BRL, Life Technologies, Inc., Gaithersburg, MD) for fewer than 14 amplified products or a Horizon 11.14 gel box for a larger number. A 1 Kb Plus DNA ladder was included in each electrophoresis run to determine the molecular size of the PCR product. Using the constant voltage mode, a field strength of 16 V/cm for the Horizon 58 horizon gel electrophoresis system gave good resolution of the PCR product after approximately 60 min. For the Horizon 11.14 system a field strength of 12 V/cm for 90 min resulted in good product separation. Progress of the PCR product through the agarose gel towards the anode was followed by the addition of 1µl of SDS loading dye 10X to every 9 µl of PCR product applied to the gel. The amplified products in the gels were stained for 20 min using ethidium bromide staining solution before being visualised under ultra violet light at a wavelength of 254 nm with a transilluminator (Alpha Innotech Corporation, San Leandro, CA). Gels were recorded with a video camera and printed to a file or a hard copy of the image was produced (Mitsubishi video copy processor, Tokyo. Japan).

2.5.1.1 Purification, quantitation and sequencing of PCR products

Materials

- Hand held ultra-violet lamp, (Ultra-Violet Prod, Inc., San Gabriel, CA.)
- Scalpel blade
- Nuclease free sterile 1.6 ml micro-tubes
- QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany)
  - QIAquick spin columns, contains a silica-gel membrane.
  - Buffer QC, contains guanidine thiocyanate as a pH indicator.
  - Buffer PE, contains ethanol
  - Buffer EB, contains 10 mM Tris-HCl, pH 8.5.
  - Collection tubes
- NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, CA)

Method

In the Dark Room, using the hand held UV lamp to visualise the correct sized PCR product, the DNA from the agarose gel was quickly excised with a scalpel and placed in a 1.6 ml microtube. The gel slice was weighed (in the microtube) and 100 µl of Buffer QG added to the tube for every 100 mg of gel. Incubation at 50°C in a heating block dissolved the gel slice in approximately 10 min and the pH was checked by the solution remaining yellow. This was important as DNA adsorption to the silica-gel membrane is only efficient at pH 7.5 or below. A QIAquick spin column was placed in a collection tube and the dissolved sample applied to the QIAquick spin column. Once loaded the column and collection tube were centrifuged for 1 min in a micro-centrifuge at ~17,900X g. The column's fluid capacity was 800 µl, so if the sample volume was greater than 800 µl the column was filled and spun, then loaded with the remainder of the sample and spun again. Flow-through was discarded from the collection tube, 500 µl of Buffer QG added to the column, centrifuged again to remove all traces of agarose and the flow-through discarded once more. The sample was washed by the addition of Buffer PE to the column, incubated for 5 min and centrifuged at ~17,900X g for a further 1 min. After discarding the flow-through the spin column was again centrifuged to remove residual wash buffer. The spin column was placed into a clean 1.6 ml microtube and the DNA eluted from the silica-gel membrane by the addition of 30 µl of Buffer EB for 1 min. Approximately 28 µl of DNA, free from the impurities of the PCR enzymatic reaction, were recovered by centrifugation of the spin column at ~17,900X g for 1 min. This DNA could now be used for direct sequencing after estimation of the concentration.

DNA concentration was estimated using a NanoDrop ND-1000 spectrophotometer which has a small footprint (23 cm x 13 cm), uses a small sample size, requires no sample dilution or cuvettes, and scans a sample through 220 – 750 nm in approximately 10 s. Surface tension of the DNA sample between two optical fibres formed a column enabling the measurement to be performed. Only 1 µl of sample was required with the spectrum and analysis being displayed on an attached computer.

Using a 2 µl micropipette 1 µl of water was placed on the measurement pedestal of the NanoDrop ND-1000 and the arm of the sample apparatus closed to draw up a column for spectrum scanning. This blanked the instrument by producing a base measurement

after which the water was wiped away with a tissue. DNA samples were applied and measured in the same manner. A hard copy of the results was available from an attached computer and printer.

The Alan Wilson Centre for Molecular Ecology and Evolution, at Massey University, carried out sequencing of the quantified samples on an ABI 3730 DNA Analyser (Applied Biosystems). Overlapping bi-directional strands of PCR product were sequenced with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

2.5.2 PCR Amplification of *Cryptosporidium* Loci

All the loci in Section 2.5.2., were chosen for their ability to discriminate between the various *Cryptosporidium* species, especially *C. hominis* and *C. parvum* cattle genotype, by PCR-RFLP.

2.5.2.1. ITS-1 rDNA PCR and RFLP

Materials; ITS-1 rDNA PCR

All primers were designed using Primer3 software (117) and manufactured by Invitrogen™ New Zealand Ltd.

- Primary primers

  Forward primer: **Cry3** 5′- AGA CAT AGG AAG GAT TGA CAG ATT G - 3′

  Primer length = 25 nt

  Primer melting temperature = 71.0°C

  GC content = 40.0%

  Reverse primer: **Cry4** 5′- CTA TTT GCG TTG AGA GAT CTG ATT G - 3′

  Primer length = 25 nt

  Primer melting temperature = 71.0°C

  GC content = 40.0%

  Product size = 1205 bp

- Secondary primers

  Forward primer: **Cry1** $^{5'}$ - TGA ATA TGC ATC GTG ATG GGG ATA G - $^{3'}$

  Primer length = 25 nt

  Primer melting temperature = 73°C

  GC content = 44.0%

  Reverse primer: **Cry2** $^{5'}$ - AGT TCA CAT TGC TTA TCG CAC TTT GC - $^{3'}$

  Primer length = 26 nt

  Primer melting temperature = 73°C

  GC content = 42.31%

  Product size = 854 bp

Materials; ITS-1 rDNA RFLP

- Restriction enzyme *Dra*1 (Invitrogen™, Carlsbad, CA) 10 units/µl

  Purified from *Deinococcus radiophilus.*

  Cleavage site $^{5'}$ - TTT ↓ AAA - $^{3'}$
  $^{3'}$ - AAA ↑ TTT - $^{5'}$

- Reaction buffer, REact® 1 Buffer 10X (Invitrogen™, Carlsbad, CA)

  Final concentration in RFLP mixture;

Tris-HCl	50 mM
$MgCl_2$	10 mM
pH	8.0

- Nuclease free distilled water
- Metaphor® agarose (BioWhittaker Molecular Applications, Rockland , ME)
- E buffer 1X
- 50 bp DNA ladder, 1 µg/µl in 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA (Invitrogen™, Carlsbad, CA)
- SDS loading dye 10X

Metaphor® agarose

A 3.5% Metaphor® agarose gel in E buffer 1X was prepared in sufficient volume to analyse all the PCR products. Before heating to dissolve, the agarose was left to sit, in E buffer 1X, at room temperature for a minimum of 30 min. Excessive aeration occurred with resulting boil over of the agarose if this precaution was not followed.

Method; ITS-1 rDNA PCR

Sterile technique was used at all times (Section 2.5.2.).

To achieve high sensitivity of ITS-1 rDNA detection and amplification, a nested PCR methodology was chosen for this locus with the primary primers designed to anneal to sequence in the conserved flanking sub-units, 18S rDNA and 5S rDNA respectively.

The PCR cocktail mixture was assembled as described in Section 2.5.2., using the ITS-1 rDNA primary primers Cry3/Cry4. Cycling conditions were; one step of 96°C for 2 min, followed by 25 cycles of 94°C for 30 s, 66°C for 30 s, and 72°C for 45 s, plus a single elongation step of 72°C for 5 min before being held at 4°C at the completion of the programme. This primary 1205 bp PCR amplified product was either used immediately as the template for the secondary PCR reaction or stored at 4°C for later amplification.

For the secondary PCR amplification the cocktail mixture and cycling conditions were identical to those used for the primary reaction with the exception of the use of the secondary primer pair, Cry1/Cry2, and the use of the primary reaction PCR product as template DNA. At the completion of the programme the PCR product was electrophoresed through a 1.6% agarose gel and stained with ethidium bromide (Section 2.5.1.). The resulting 854 bp PCR product was either subjected to RFLP analysis to establish genotype or purified and quantitated for direct sequence analysis.

Optimisation of the PCR cycling conditions, temperature (temperature gradient PCR and touchdown PCR), $MgCl_2$ strength, and cycle number, were established before the method was put into routine use.

Method; ITS-1 rDNA RFLP

Sterile technique was used at all times (Section 2.5.2.).

The restriction endonuclease digestion reactants were assembled in 0.2 ml thin-walled thermo-tubes and incubated at 37°C for 3 hours. Each RFLP reaction contained;

Secondary PCR product	10 µl
REact® 1 Buffer 10X	2 µl
*Dra*1 restriction enzyme (10 Units/µl)	2 µl
Nuclease free sterile water	6 µl

The restriction endonuclease digestion fragments were resolved by electrophoresis in a 3.5% Metaphor® agarose gel using the 50 bp DNA ladder, under the same conditions found in Section 5.2.1.

2.5.2.2 18S rDNA PCR and RFLP

Materials; 18S rDNA PCR

All primers were designed using Primer3 software (117) and manufactured by Invitrogen™ New Zealand Ltd.

- Primary primers

  Forward primer: **18S L1** $^{5'}$ - GTT AAA CTG CGA ATG GCT CA - $^{3'}$

  Primer length = 20 nt

  Primer melting temperature = 66°C

  GC content = 45.0%

  Reverse primer: **18S L2** $^{5'}$ - CCA TTT CCT TCG AAA CAG GA - $^{3'}$

  Primer length = 20 nt

  Primer melting temperature = 66°C

  GC content = 45.0%

  Product size = 1397 bp

- Secondary primers

  Forward primer: **18S S1** $^{5'}$ - CTC GAC TTT ATG GAA GGG TTG - $^{3'}$

  Primer length = 21 nt

  Primer melting temperature = 69°C

  GC content = 47.62%

Reverse primer: **18S S2** $^{5'}$ - CCT CCA ATC TCT AGT TGG CAT A -$^{3'}$

Primer length = 22 nt

Primer melting temperature = 69°C

GC content = 45.45%

Product size = 832 bp

Materials; 18S rDNA RFLP

- Restriction enzyme *Ssp*1 (Invitrogen™, Carlsbad, CA) 10 Units/µl
  Purified from *Sphaerotilus* sp. ATCC® 13925

  Cleavage site $^{5'}$ - AAT ↓ ATT - $^{3'}$
  $^{3'}$ - TTA ↑ TAA - $^{5'}$

- Reaction buffer, REact® 6 Buffer 10X (Invitrogen™, Carlsbad, CA).
  Final concentration in RFLP mixture;

Tris-HCl	50 mM
$MgCl_2$	6 mM
NaCl	50 mM
KCl	50 mM
pH	7.4

- Restriction enzyme *Vsp*1 (Promega, Madison, WI) 10 Units/µl

  Cleavage site $^{5'}$ - AT ↓ TA AT - $^{3'}$
  $^{3'}$ - TA AT ↑ TA - $^{5'}$

- Buffer D 10X (Promega, Madison, WI)
  Final concentration in RFLP mixture;

Tris-HCl	6 mM
MgCl	6 mM
NaCl	150 mM
pH	7.9

- Bovine serum, acetylated 100X, 10 mg/ml (Promega, Madison, WI)
- Nuclease free distilled water
- Metaphor® agarose (BioWhittaker Molecular Applications, Rockland , ME)
- E buffer 1X
- 50 bp DNA ladder, 1 µg/µl in 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA (Invitrogen™, Carlsbad, CA)
- SDS loading dye 10X

Metaphor® agarose

See Section 2.5.2.1.

Method; 18S rDNA PCR

Sterile technique was used at all times (Section 2.5.2.).

Sensitivity of 18S rDNA detection and amplification were assured by using a nested PCR technique with the secondary primers designed within conserved sequence to flank several highly polymorphic regions within this locus.

The PCR cocktail mixture was assembled as described in Section 2.5.2., using the 18S rDNA primary primer pair 18S L1/18S L2. Cycling conditions were; one step of 96°C for 2 min, followed by 25 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 45 s, plus a single elongation step of 72°C for 5 min before being held at 4°C at the completion of the programme. This primary 1397 bp PCR amplified product was either used immediately as the template for the secondary PCR reaction or stored at 4°C for later amplification.

For the secondary PCR amplification the cocktail mixture and cycling conditions were identical to those used for the primary reaction with the exception of the use of the secondary primer pair, 18S S1/18S S2, and the use of the primary PCR product as template DNA. At the completion of the programme the PCR product was electrophoresed through a 1.6% agarose gel and stained with ethidium bromide (Section 2.5.1.). The resulting 832 bp PCR product was either subjected to RFLP analysis to establish genotype or purified and quantitated for direct sequence analysis.

Optimisation of the PCR cycling conditions, temperature (temperature gradient PCR and touchdown PCR), $MgCl_2$ strength, and cycle number were established before the method was put into routine.

Method; 18S rDNA RFLP

Sterile technique was used at all times (Section 2.5.2.).

Differentiation of *C. hominis* from *C. parvum* bovine genotype was made using the restriction enzyme *Vsp*1 while other *Cryptosporidium* species were differentiated using *Ssp*1.

The restriction endonuclease digestion reactants were assembled in 0.2 ml thin-walled thermo-tubes and incubated at 37°C for 3 hours. The two RFLP reactions contained either;

PCR product	10 µl
BSA (10mg/ml)	0.2 µl
REact® 6 Buffer 10X (*Ssp*1)	2 µl
*Ssp*1 restriction enzyme (10 Units/µl)	2 µl
nuclease free sterile water	5.8 µl

or

PCR product	10 µl
BSA (10mg/ml)	0.2 µl
Buffer D 10X (*Vsp*1)	2µl
*Vsp*1 restriction enzyme (10 Units/µl)	2 µl
nuclease free sterile water	5.8 µl

The restriction endonuclease digestion fragments were resolved by electrophoresis in a 3.5% Metaphor® agarose gel using the 50 bp DNA ladder, under the same conditions found in Section 5.2.1.

2.5.2.3 β-tubulin PCR and RFLP

This method followed closely the publication of Caccio *et al.* (1999) with minor modifications to the PCR cocktail mixture, cycling programme and medium used for the RFLP fractionation (19).

Materials; β-tubulin PCR

- PCR cocktail mixture; see Section 2.5.1.
- Primers (Invitrogen™, Carlsbad, CA)

  Forward primer: **Tub1** $^{5'}$ - ATG AGA GAA ATT GTT CAT GTT - $^{3'}$

  Primer length = 21 nt

  Primer melting temperature = 61°C

  GC content = 28%

  Reverse primer: **Tub2** $^{5'}$ - AAA GGT CTG CAA AAT ACG ATC - $^{3'}$

  Primer length = 21 nt

  Primer melting temperature = 65°C

  GC content = 38%

  Product size = 592 bp

Materials; β-tubulin RFLP

- Restriction enzyme *Dde*1 (Invitrogen™, Carlsbad, CA) 10 units/µl
  Purified from *Desulfovibrio desulfuricans* NCIB Norway

  Cleavage site $^{5'}$ - C ↓ TNA G - $^{3'}$

  $^{3'}$ - G ANT ↑ C - $^{5'}$

- Reaction buffer, React® 3 Buffer 10X (Invitrogen™, Carlsbad, CA).
  Final concentration in RFLP mixture;

Tris-HCl	50 mM
$MgCl_2$	10 mM
NaCl	100 mM
pH	8.0

- Nuclease free distilled water
- Metaphor® agarose (BioWhittaker Molecular Applications, Rockland , ME)
- E buffer 1X
- 50 bp DNA ladder, 1 μg/μl in 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA (Invitrogen™, Carlsbad, CA)
- SDS loading dye 10X

Metaphor® agarose

See Section 2.5.2.1.

Method; β-tubulin PCR

Sterile technique was used at all times (Section 2.5.2.).

The PCR cocktail mixture was assembled as described in Section 2.5.2., using the primer pair Tub1/Tub2. Cycling conditions were; one step of 96°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s, plus a single elongation step of 72°C for 7 min before being held at 4°C at the completion of the programme. At the completion of the programme the PCR product was electrophoresed through a 1.6% agarose gel and stained with ethidium bromide (Section 2.5.1.). The resulting 592 bp PCR product was either subjected to RFLP analysis to establish genotype or purified and quantitated for direct sequence analysis.

Method; β-tubulin RFLP

Sterile technique was used at all times (Section 2.5.2.).

The restriction endonuclease digestion reactants were assembled in 0.2 ml thin-walled thermo-tubes and incubated at 37°C for 3 hours. Each RFLP reaction contained;

PCR product	8 μl
REact® 3 Buffer 10X	2 μl
*Dde*1 restriction enzyme (10 Units/μl)	1μl
nuclease free sterile water	9 μl

The restriction endonuclease digestion fragments were resolved by electrophoresis in a 3.5% Metaphor® agarose gel using the 50 bp DNA ladder, under the same conditions found in Section 5.2.1.

2.5.2.4 Poly-T PCR and RFLP

This method followed closely the publication of Carraway *et al.* (1997) with minor modifications to the PCR cocktail mixture and cycling programme (27).

Materials; Poly-T PCR

- PCR cocktail mixture; see Section 2.5.2.
- Primers (Invitrogen™, Carlsbad, CA)

  Forward primer: **Poly-T1** $^{5'}$ - CTC TTA ATC CAA TCA TTA CAA C - $^{3'}$

  Primer length = 22 nt

  Primer melting temperature = 64°C

  GC content = 31%

  Reverse primer: **Poly-T2** $^{5'}$ - GAG TCT AAT AAT AAA CCA CTG - $^{3'}$

  Primer length = 21

  Primer melting temperature = 63°C

  GC content = 33%

  Product size = 318 bp

Materials; Poly-T RFLP

- Restriction enzyme *Rsa*1 (Invitrogen™, Carlsbad, CA) 10 units/µl

  Purified from *Rhodopseudomonas sphaeroides*

  Cleavage site $^{5'}$ - GT ↓ AC - $^{3'}$

  $^{3'}$ - CA ↑ T G - $^{5'}$

- Reaction buffer, React® 1 Buffer 10X (Invitrogen™, Carlsbad, CA).

  Final concentration in RFLP mixture;

  Tris-HCl 50 mM

$MgCl_2$	10 mM
pH	8.0

- Nuclease free distilled water
- Metaphor® agarose (BioWhittaker Molecular Applications, Rockland , ME)
- E buffer 1X
- 50 bp DNA ladder, 1 µg/µl in 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA (Invitrogen™, Carlsbad, CA)
- SDS loading dye 10X

Metaphor® agarose

See Section 2.5.2.1.

Method; Poly-T PCR

Sterile technique was used at all times (Section 2.5.2.).

The PCR cocktail mixture was assembled as described in Section 2.5.2., using the primer pair Poly-T1/Poly-T2. Cycling conditions were; one step of 96°C for 2 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 45 s, plus a single elongation step of 72°C for 5 min before being held at 4°C at the completion of the programme. At the completion of the programme the PCR product was electrophoresed through a 1.6% agarose gel and stained with ethidium bromide (Section 2.5.1.). The resulting 318 bp PCR product was either subjected to RFLP analysis to establish genotype or purified and quantitated for direct sequence analysis.

Method; Poly-T RFLP

Sterile technique was used at all times (Section 2.5.2.).

The restriction endonuclease digestion reactants were assembled in 0.2 ml thin-walled thermo-tubes and incubated at 37°C for 3 hours. Each RFLP reaction contained;

PCR product	8 µl
REact® 1 Buffer 10X	2 µl
*Rsa*1 restriction enzyme (10 Units/µl)	1 µl

nuclease free sterile water 9 µl

The restriction endonuclease digestion fragments were resolved by electrophoresis in a 3.5% Metaphor® agarose gel using the 50 bp DNA ladder, under the same conditions found in Section 5.2.1.

2.5.2.5 COWP PCR and RFLP

This method followed closely the publication of Spano *et al.* (1997) with minor modifications to the PCR cocktail mixture, cycling programme and medium used for the RFLP fractionation (125).

Materials; COWP PCR

- PCR cocktail mixture; see Section 2.5.2.
- Primers (Invitrogen™, Carlsbad, CA)

  Forward primer: **COWP1** $^{5'}$ - GTA GAT AAT GGA AGA GAT TGT G - $^{3'}$

  Primer length = 22 nt

  Primer melting temperature = 66°C

  GC content = 36%

  Reverse primer: **COWP2** $^{5'}$ - GGA CTG AAA TAC AGG CAT TAT CTT G - $^{3'}$

  Primer length = 25 nt

  Primer melting temperature = 71°C

  GC content = 40%

  Product size = 550 bp

Materials; COWP RFLP

- Restriction enzyme *Rsa*1 (Invitrogen™, Carlsbad, CA) 10 units/µl

  Purified from *Rhodopseudomonas sphaeroides*

  Cleavage site $^{5'}$ - GT ↓ AC - $^{3'}$

  $^{3'}$ - CA ↑ T G - $^{5'}$

- Reaction buffer, React® 1 Buffer 10X (Invitrogen™, Carlsbad, CA).

  Final concentration in RFLP mixture;

Tris-HCl	50 mM
$MgCl_2$	10 mM
pH	8.0

- Nuclease free distilled water
- Metaphor® agarose (BioWhittaker Molecular Applications, Rockland , ME)
- E buffer 1X
- 50 bp DNA ladder, 1 µg/µl in 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA (Invitrogen™, Carlsbad, CA)
- SDS loading dye 10X

Metaphor® agarose

See Section 2.5.2.1.

Method; COWP PCR

Sterile technique was used at all times (Section 2.5.2.).

The PCR cocktail mixture was assembled as described in Section 2.5.2.using the primer pair COWP1/COWP2. Cycling conditions were; one step of 96°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s, plus a single elongation step of 72°C for 7 min before being held at 4°C at the completion of the programme. At the completion of the programme the PCR product was electrophoresed through a 1.6% agarose gel and stained with ethidium bromide (Section 2.5.1.). The resulting 550 bp PCR product was either subjected to RFLP analysis to establish genotype or purified and quantitated for direct sequence analysis.

Method; COWP RFLP

Sterile technique was used at all times (Section 2.5.2.).

The restriction endonuclease digestion reactants were assembled in 0.2 ml thin-walled thermo-tubes and incubated at 37°C for 3 hours. Each RFLP reaction contained;

PCR product	8 µl
REact® 1 Buffer 10X	2 µl
*Rsa*1 restriction enzyme (10 Units/µl)	1 µl

nuclease free sterile water 9 µl

The restriction endonuclease digestion fragments were resolved by electrophoresis in a 3.5% Metaphor® agarose gel using the 50 bp DNA ladder, under the same conditions found in Section 5.2.1.

2.5.2.6 RNR PCR and RFLP

This method followed closely the publication of Widmer *et al.* (1998) with minor modifications to the PCR cocktail mixture, cycling programme and medium used for the RFLP fractionation (147).

Materials; RNR PCR

- PCR cocktail mixture; see Section 2.5.2.
- Primers (Invitrogen™, Carlsbad, CA)

  Forward primer: **RNR1** $^{5'}$ - GAC CTA TTG TTT CAA GTA AC - $^{3'}$

  Primer length = 20 nt

  Primer melting temperature = 62°C

  GC content = 41%

  Reverse primer: **RNR2** $^{5'}$ - GTA AAA TAC CCT TAC TCT CAG GCG - $^{3'}$

  Primer length = 24 nt

  Primer melting temperature = 72°C

  GC content = 45%

  Product size = 441 bp

Materials; RNR RFLP

- Restriction enzyme *Tsp*509 I (New England Biolabs, Beverly, MA) 10 units/µl

  Purified from *Thermus* species ITI 346

  Cleavage site $^{5'}$ - ↓ AATT - $^{3'}$

  $^{3'}$ - TTAA ↑ - $^{5'}$

- Reaction buffer, NEBuffer 1 (New England Biolabs,Beverly, MA)

  Final concentration in RFLP mixture;

Bis Tris-Propane-HCl	10 mM
$MgCl_2$	10 mM
Dithiothreitol (DTT)	1 mM
pH	7.0

- Nuclease free distilled water
- Metaphor® agarose (BioWhittaker Molecular Applications, Rockland , ME)
- E buffer 1X
- 50 bp DNA ladder, 1 µg/µl in 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA (Invitrogen™, Carlsbad, CA)
- SDS loading dye 10X

Metaphor® agarose

See Section 2.5.2.1.

Method; RNR PCR

Sterile technique was used at all times (Section 2.5.2.).

The PCR cocktail mixture was assembled as described in Section 2.5.2., using the primer pair RNR1/RNR2. Cycling conditions were; one step of 96°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s, plus a single elongation step of 72°C for 7 min before being held at 4°C at the completion of the programme. At the completion of the programme the PCR product was electrophoresed through a 1.6% agarose gel and stained with ethidium bromide (Section 2.5.1.). The resulting 441 bp PCR product was either subjected to RFLP analysis to establish genotype or purified and quantitated for direct sequence analysis.

Method; RNR RFLP

Sterile technique was used at all times (Section 2.5.2.).

The restriction endonuclease digestion reactants were assembled in 0.2 ml thin-walled thermo-tubes and incubated at 65°C for 3 hours. Each RFLP reaction contained;

PCR product	8 µl
NEBuffer 1 10X	2 µl

Tsp 509 1 restriction enzyme (10 Units/µl)	1µl
nuclease sterile water	9 µl

The restriction endonuclease digestion fragments were resolved by electrophoresis in a 3.5% Metaphor® agarose gel using the 50 bp DNA ladder, under the same conditions found in Section 5.2.1.

2.5.3 PCR sensitivity of specific *Cryptosporidium* loci

To check the PCR sensitivity of each of the above loci, *C. hominis* oocysts were isolated by IMS, the concentration adjusted to 10^5 and triplicate serial ten fold dilutions made in 200 µl of TE buffer containing 1% Nonidet P40. The range of the dilution series was 10^5 to 10^{-1} oocysts. The nucleic acid was extracted (Section 2.4.1.) and the various individual loci amplified by PCR (Sections 2.5.2.1. to 2.5.2.6.). PCR products were electrophoresed, visualised and the positive results out of three recorded (Section 2.5.1.).

2.6 RT-PCR OF VIRAL-LIKE dsRNA

Immuno-competent humans are usually only parasitised by either *C. hominis* or *C. parvum* bovine genotype and until recently there have been no tools available that were capable of intra-genotype/species genetic differentiation. This technique examined the intraspecies sequence diversity in the two viral-like RNA molecules found in the cytoplasm of *C. hominis* and *C. parvum* bovine genotype and looked at their potential to investigate cryptosporidiosis outbreaks.

In the reverse transcription reaction a primer was annealed to RNA and elongated by reverse transcriptase to form a cDNA molecule. This cDNA then served as a template for subsequent amplification by PCR.

2.6.1 dsRNA purification

Cryptosporidium oocysts from human faecal specimens were isolated, washed and the nucleic acid extracted as described in Sections 2.3 and 2.4. Any oocysts that could not

be processed immediately were stored in 400 µl of RNA*later*™ (Ambion, Austin TX) at 4°C. These stored oocysts, when ready to be processed, were washed twice in TE buffer + 1% Nonidet P40 and the nucleic acid extracted. Genomic *Cryptosporidium* DNA was removed to ensure that any PCR product from the RT-PCR was derived from cDNA.

Materials

DNA-*free*™ kit (Ambion, Austin, TX)

- DNase I, RNase- free 2 Units/µl
- DNase I Buffer 10X

Tris-HCl	100 mM
$MgCl_2$	25 mM
$CaCl_2$	1 mM
pH	7.5

- DNase Inactivation Reagent, provided as a 10 – 20% slurry in water/EDTA
- Nuclease-free water/0.1 mM EDTA

Method

A 50 µl aliquot of oocyst nucleic acid extract was treated with 5 µl of DNase I Buffer 10X and 1 µl of DNase I at 37°C for 30 min. The DNase Inactivation Reagent was resuspended and 5 µl added to the sample for a 2 min incubation at room temperature. Centrifugation at ~17,900 x g for 1 min pelleted the DNase Inactivation Reagent and the DNA free supernatant was removed to a new microtube for storage. The DNase Inactivation Reagent in addition to removing DNase I also removes divalent cations, which can catalyse heat-mediated degradation of RNA at first step in the RT-PCR process.

2.6.2 dsRNA RT-PCR

All RT-PCR reactions were performed using a RETROscript™ (Ambion, Austin, TX) kit. This kit allows the RT-PCR reaction to be performed in different ways depending on the user's preference, e.g., either random primers (kit) or specific primers for use as first strand primers, and either two step or one step RT-PCR.

The two step RT-PCR method with random decamers as primers was used for the first step and the primers of Khramstov *et al.* (2000) in the second step (64). The latter primers, two different forward primers and a common reverse primer, amplified two differing lengths of the smaller of the two viral-like extrachromosomal RNA molecules found in *Cryptosporidium.*

Materials

- *Cryptosporidium* oocyst RNA (DNA free)
- RETROscript™ kit

RT Buffer 10X	500 mM Tris-HCl, pH 8.3
	750 mM KCl
	30 mM $MgCl_2$
	50 mM DTT
PCR Buffer 10X	100 mM Tris-HCl, pH 8.3
	500 mM KCl
	15 mM $MgCl_2$
dNTP mix	2.5 mM each dNTP
Random Decamers	50 µM
Oligo (dT) Primers	50 µM
MMLV-RT*	100 Units/µl
RNase Inhibitor	10 Units/µl
Nuclease-free Water	
Control PCR Primers	5 µM each, forward and reverse
Control Template RNA	0.5 mg/ml mouse liver RNA
High Resolution Gel Loading Solution 5X	

* Moloney-Murine Leukemia Virus Reverse Transcriptase

- Primers (Invitrogen™, Carlsbad, CA)
  Stock solution of 1 mM. Diluted to a working strength of 20 µM.

  First forward primer: **dsRNA-2F** $^{5'}$ - CAT GGA GGT TCA TGT AGT ACC - ^{3}
  Primer length = 21 nt
  Primer melting temperature = 54.5°C
  GC content = 47.62%

Second forward primer: **dsRNA-1F** 5′ - TGC AGT TTA CTA TCC AGT GG - 3′

Primer length = 20 nt

Primer melting temperature = 53.83°C

GC content = 45%

Reverse primer: **dsRNA-R** 5′ - GCA GAA GGG TTC TAT GAT TC - 3′

Primer length = 20 nt

Primer melting temperature = 53.9°C

GC content = 45%

First product size = 261 bp

Second product size = 173 bp

Method

Sterile technique was used at all times (Section 2.5.2.).

- Reverse transcription reaction:

A two step RT-PCR was used for all reactions and performed in 0.2 ml thin-walled thermo-tubes with flat caps (Abgene®, Surrey, England). To 10 µl of oocyst RNA, 2 µl of random decamers was added, mixed, spun briefly and heated at 70°C for 3 min before being spun briefly again and placed on ice. A positive RT control was included in each batch, consisting of 1 µl of control template RNA (mouse liver RNA), 2 µl of random decamers and 9 µl of nuclease-free water. This control was heat denatured as above. The remaining RT reagents were added to the heat denatured RNA, with each reaction receiving;

RT Buffer 10X	2 µl
dNTP mix	4 µl
RNase Inhibitor	1 µl
Reverse transcriptase*	1 µl
Final volume	20 µl

*Reverse transcriptase was used diluted 1:10 in RT Buffer 1X although it could be used neat.

The mixture was gently mixed, spun briefly and incubated at 43°C for 1 hr. To inactivate the reverse transcriptase the temperature was increased to 92°C for 10 min. If the PCR was not going to be performed immediately the reactions were stored at -20°C.

- PCR reaction

Three controls were included in each batch of samples processed.

1. Positive control. Control PCR Primers from the RETROscript™ kit were substituted for the test primers in this second step with the Control Template RNA. These primers amplified a 361 bp highly conserved region of the mouse S15 housekeeping gene and span two introns.

2. Minus-RT control. To check that the template for the PCR reaction was cDNA and not genomic DNA, a minus-RT control was included but in an effort to be economically, rather than carry out a mock reverse transcription using all the reagents excluding the reverse transcriptase, RNA was simply added to the PCR as a template. Any PCR product from the RNA would have to come from contaminating DNA, as RNA cannot be amplified by PCR.

3. Minus-template control. To check that none of the PCR reagents were contaminated with DNA all the PCR reagents were assembled but water was used as the template rather than cDNA.

All reactions were carried out in new 0.2 ml thin-walled thermo-tubes with flat caps (Abgene®, Surrey, England). For each RT cDNA sample, two PCR reactions were performed; one with the primers dsRNA-2F and dsRNA-R and one with the primers dsRNA-1F and dsRNA-R. The following mixture was assembled for each reaction;

RT reaction cDNA	2 µl
PCR Buffer 10X	2 µl
dNTP mixture	1 µl
Appropriate primers	1 µl of each
Taq polymerase (5 units/µl)	0.5 µl
Nuclease free water	12.5 µl

Cycling conditions were; one step of 96°C for 2 min, followed by 35 cycles of 94°C for 30 s, 51°C for 30 s and 72°C for 30 s, plus a single elongation step of 72°C for 5 min before being held at 4°C at the completion of the programme. The amplified PCR product was electrophoresed and stained with ethidium bromide (Section 2.5.1.) and if a PCR product of the right size was present it was purified and quantitated for direct sequence analysis. The 261 bp or 173 bp PCR product was stored at 4°C.

2.7 DATA ANALYSIS

2.7.1 Alignment of *Cryptosporidium* DNA sequences

Sequence data received from the Alan Wilson Centre for Molecular Ecology and Evolution was edited and assembled using Perkin Elmer's MT Navigator PPC software, version 1.0.2b3 (Applied Biosystems). The DNA sequence alignments were generated with ClustalX, version 1.8 (136).

2.7.2 Phylogenetic analysis of aligned *Cryptosporidium* DNA sequences

Aligned sequence data from animals and humans at the 18S rDNA locus, including selected animal sequences from GenBank for the sake of comparison, were put into a format able to be published, using software developed by Kumar *et al.* (2001) - MEGA2, version 2.1 (Molecular Evolutionary Genetic Analysis) (67). The aligned sequence data from the dsRNA was treated in an identical manner.

Phylogenetic trees of the 18S rDNA and dsRNA sequence data were generated using PAUP for MacIntosh™ software, version 4.0b10 (133). The trees were assembled using the neighbour-joining method with genetic distances calculated by the general time-relative model. Branch reliability of the trees were tested by bootstrap values obtained from 1,000 pseudo-replications, using *C. serpentis* as an outgroup for the 18S rDNA locus and *C. parvum* bovine genotype KSU-1 for the dsRNA samples.

2.7.3 Nucleotide sequence accession numbers

Published 18S rDNA sequences used for phylogenetic analysis included *C. andersoni* [AB089285], *C. baileyi* [AF093495] (150), *C. canis* [AF112576] (153), *C. felis* [AF112575] (153), *C. meleagridis* [AF112574] (153), *C. muris* [AF093498] (150), *C. hominis* [AF093489] (150), *C. parvum* bovine genotype [AF108864] (96), *C. parvum* pig genotype [AF115377] (153), *C. serpentis* [AF093502] (150), and *C. wrairi* [AF115378] (153). Two novel *Cryptosporidium* sequences and a *C. parvum* rabbit genotype sequence used to interpret results have been deposited with GenBank under the accession numbers AY458612 to AY458614 (70).

CHAPTER 3: RESULTS

3.1 COMPARISON OF STAINING TECHNIQUES AND MICROSCOPY

Direct immunofluorescent antibody stain (DFA) methodology was found to be superior to the traditional modified Ziehl-Nelseen acid fast stain (ZN) for detecting oocysts in animal faeces. Although the modified ZN acid fast stain was inexpensive to perform it lacked the specificity and sensitivity of the DFA technique. Great care was needed with the decolourization step of the modified ZN technique as over decolourization resulted in false negative readings, while under decolourization gave false positive results with yeast cells retaining the basic fuchsin. The time used to decolourise the smear was a subjective decision that depended on the thickness of the faecal smear. By diluting the monoclonal FITC conjugate tenfold the expense of the DFA technique was offset without a loss in sensitivity. Reading the modified ZN stained smears required up to 15 min per slide at 320X magnification, as opposed to the 5 min needed to thoroughly scan a DFA stained smear at 125X magnification. The DFA stained smears had a dark background contrasting with the bright apple green fluorescently stained oocysts (Figure 2A.). This was in contrast to the "busy" background of the modified ZN stained smears (Figure 2B.).

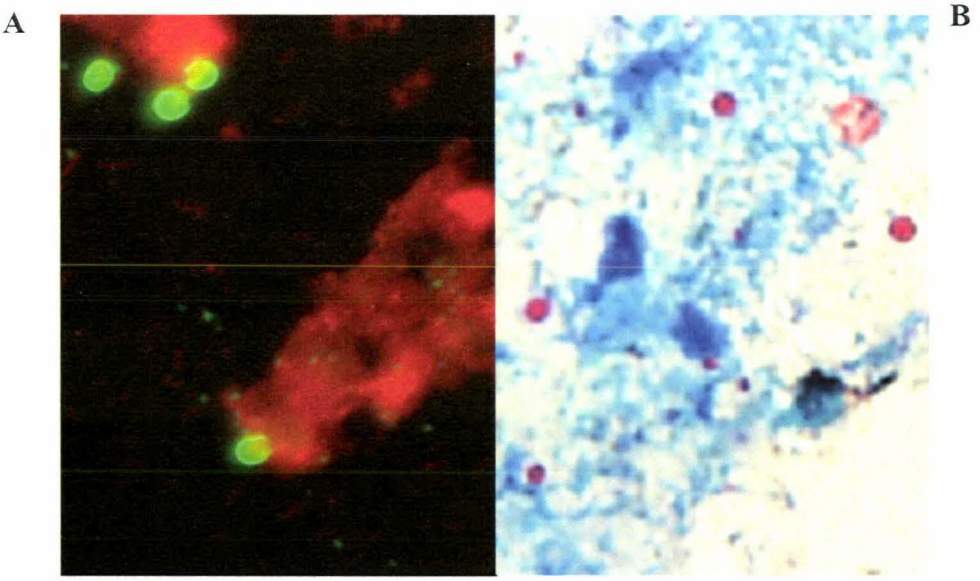


Figure 2. DFA (A) and modified ZN (B) of the same *Cryptosporidium* positive faecal sample at 640X magnification.

DIC microscopy was superior to phase contrast microscopy for assessing the relative health of the isolated oocysts. Time and expense were not factors in the comparison but the DIC microscopy gave greater resolution of the oocysts internal structures (Figure 3.).

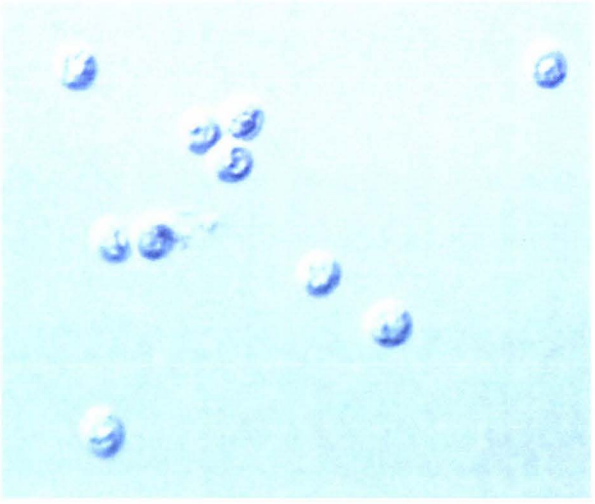


Figure 3. Normaski DIC photomicrograph of viable *C. hominis* oocysts after IMS isolation. 800X magnification.

3.2 SPECIATING/GENOTYPING *CRYPTOSPORIDIUM* ISOLATES AT VARIOUS LOCI

Cryptosporidium is a diverse genus with many animal hosts, each of which may harbour a species or genotype specific to it. A locus, for genetic characterisation, had to be selected which would give good inter-species/genotype resolution while having the ability to be amplified from a small number of oocysts. As humans are almost exclusively infected with either *C. hominis* or *C. parvum* bovine genotype, the locus selected had to demonstrate good differentiation between the two. Those methods targeting the rDNA were most sensitive, as each sporozoite contains five rDNA copies as opposed to the single copies of the β-tubulin, COWP and RNR genes and the poly threonine repeat motif (Table 7.). Each oocyst therefore, contained 20 rDNA genes while the other loci number only four genes per oocyst. Coupled with a nested primer technique, the rDNA methods were more sensitive but had the problem of being labour intensive and susceptible to contamination through the smallest flaw in sterile technique.

Table 7. PCR sensitivity at six different loci. Each oocyst concentration was tested in triplicate.

	Cryptosporidium oocyst concentration (volume 200 µl)					
	10^5	10^4	10^3	10^2	10^1	10^0
ITS-1 rDNA	3/3*	3/3	3/3	3/3	3/3	0/3
18S rDNA	3/3	3/3	3/3	3/3	3/3	0/3
β-tubulin	3/3	3/3	3/3	3/3	2/3	0/3
Poly T	3/3	3/3	3/3	3/3	2/3	0/3
COWP	3/3	3/3	2/3	0/3	0/3	0/3
RNR	3/3	3/3	0/3	0/3	0/3	0/3

*Triplicate tests with positives out of three recorded.

3.2.1 ITS-1 rDNA PCR-RFLP

The ITS-1 rDNA was originally chosen to show that species and genotypes could be differentiated at this locus as reported by Morgan *et al.* (1999) (88). Factors influencing this choice of locus were the conserved regions surrounding the polymorphic ITS-1 rDNA and the large range in the locus base pair length between *Cryptosporidium* isolates from different hosts (Table 3.). It also provided good differentiation between *C. hominis* and *C. parvum* bovine genotype through the *Dra*1 restriction enzyme sites, as predicted from published ITS-1 rDNA sequences (Figure 4. and Table 4.).

Initial results were promising with a large amount of PCR product being amplified from isolated oocysts, however mis-priming due to the high AT content of the ITS-1 rDNA resulted in many non-specific bands being produced. Morgan *et al.* (1999) reported the ITS-1 rDNA AT content of *C. hominis* to be 91% and *C. parvum* bovine genotype to be 88% (88). The results of mis-priming are illustrated in Figure 5.; with lanes 3, 5, and 7 having six non-specific bands plus the target band, lanes 2, 4, and 6 having five non-specific bands, lane 10 having four, lane 8 having two, whilst lane 9 consists of a single target band. A comparison of the amount of amplified target DNA in lanes 3, 5, and 7 with the single amplified band in lane 9, suggests that the mis-priming was related to the concentration of DNA extracted from the oocysts and therefore the number of sporozoite containing oocysts in the faecal sample.

Figure 4. *C. parvum* KSU-1 (bovine genotype) and *C. hominis* GenBank AF 090312 aligned partial rRNA gene sequences showing the primary forward and reverse primers highlighted in blue, the secondary forward and reverse primers highlighted in green and the *Dra*1 restriction enzyme cleavage sites highlighted in yellow.

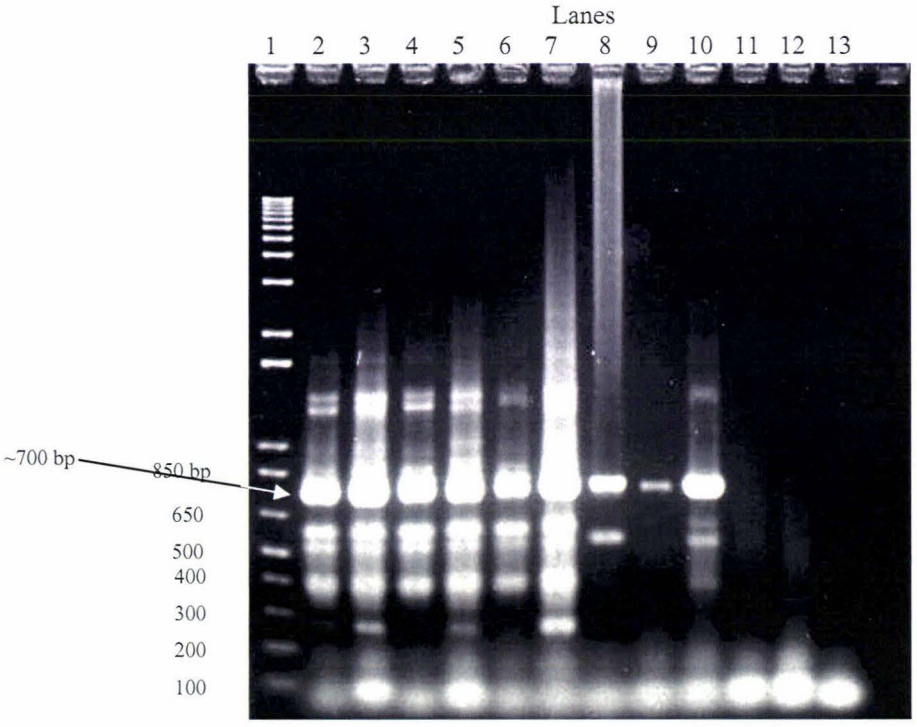


Figure 5. ITS-1 rDNA PCR of human and bovine faecal *Cryptosporidium* isolates. Lane 1, 1 Kb plus DNA ladder; Lanes 2 to 5, positive human samples; Lanes 6 to 10 positive bovine samples; Lane 11, negative bovine sample; Lane 12, primary PCR negative control; Lane 13, secondary PCR negative control.

The target PCR product sizes were 885 bp for *C. hominis* and 854 bp for *C. parvum* bovine genotype but possible conformational changes during electrophoresis resulted in the product appearing to be approximately 700 bp in length. Digestion of the target band with the restriction endonuclease *Dra*1, after excision and extraction from the gel, produced fragment sizes which totalled the correct number of base pairs for their respective species (Figure 6.).

Although PCR-RFLP results were obtained at this locus by restriction endonuclease digestion, the fragment patterns were interpreted with caution, as non-specific bands were often present. Correct fragment sizes for *C. hominis* were 349, 174, 132, 126 and 104 bp, whereas *C. parvum* bovine genotype restriction fragment sizes were 614 and 240 bp (Figure 6.). As the restriction digests were electrophoresed through 3.5% Metaphor® agarose gel there was good resolution between fragments of similar size.

Direct ITS-1 rDNA sequencing of the PCR product was not successful due to the high AT content of this locus. Approximately 350 bases could be sequenced from the 5′ prime end before the first group of tandem repeat thiamine bases disrupted the sequence, while only 150 bases could be sequenced from the 3′ prime end before long series of repeat adenine bases broke up the sequence.

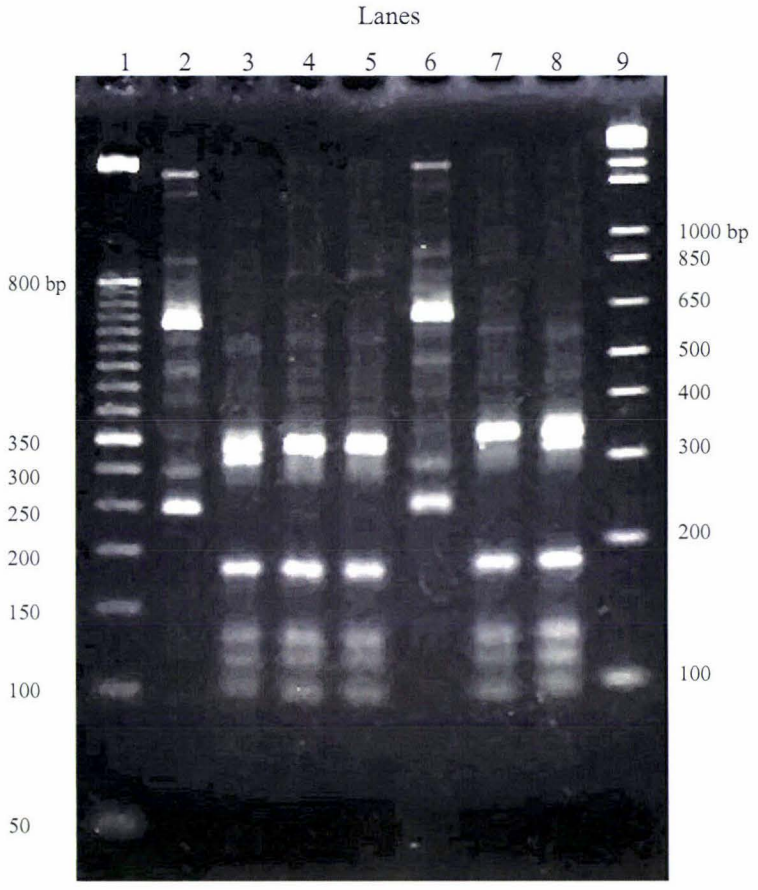


Figure 6. PCR-RFLP gel of ITS-1 rDNA digested with *Dra*1. Lane 1, 50 bp DNA ladder; lane 2, *C. parvum* bovine genotype; lanes 3, 4, and 5, *C. hominis*; lane 6, *C. parvum* bovine genotype; lanes 7 and 8, *C. hominis*; lane 9, 1 Kb plus DNA ladder.

The sensitivity of the nested primer PCR technique was capable of detecting between one and ten oocysts (Table 7.) but the method required double handling and vigilance against contamination with extraneous DNA.

Of the 1169 faecal specimens examined by DFA, only 95 (8%) human and 38 (3%) bovine faecal specimens were found to contain *Cryptosporidium* oocysts with the

exception of a single rabbit faecal sample (Table 8.). The rabbit isolate, on ITS-1 rDNA PCR-RFLP, had a *Dra*1 fragment pattern identical to *C. hominis*. It was later found, by 18S rDNA sequencing, that this sample was a unique *C. parvum* genotype not before documented. Subsequently the 18S rDNA sequence has been published by three authors (70, 118, 156).

Table 8. Total number of faecal samples tested by DFA for the presence of *Cryptosporidium* oocysts from various hosts. Positive samples were speciated by ITS-1 rDNA PCR-RFLP.

Host	Total samples	*C. hominis*	*C. parvum* bovine
Human	141	46	49
Calf	318	0	34
Cattle	564	0	4
Sheep	21	0	0
Dog	1	0	0
Cat	1	0	0
Rabbit*	22	0	0
Hare	13	0	0
Duck	20	0	0
Possum	20	0	0
Sea Lion	10	0	0
Sea Lion Pup	9	0	0
Fur Seal	10	0	0
Hector's Dolphin	10	0	0
Common Dolphin	1	0	0
Marine Mammal	8	0	0
Total	1169	46	87

*One rabbit isolate genotyped as *C. hominis* by ITS-1 rDNA PCR-RFLP but on sequencing the 18S rDNA it belonged to a genotype unique to the rabbit. GenBank number AY458614 (70).

C. hominis and *C. parvum* bovine genotype were found in 33% and 35% respectively of the human faecal specimens faecal specimens examined. The time period over which these specimens were collected ranged from late August 2000 to late June 2001. All the human faecal specimens analysed during the spring contained *C. parvum* bovine genotype and those collected during late summer/autumn contained *C. hominis* (72). This change in transmission cycle, coinciding with the change in season was again

apparent when genotyping humans isolates at the 18S rDNA and β-tubulin loci (Figure 13.).

The cattle examined were exclusively infected with *C. parvum* bovine genotype. While 11% of calves' faecal samples contained oocysts only 0.7% of adult cattle did, corresponding with the observation that *Cryptosporidium* infection is a disease of young animals. Samples were collected from the calves during the spring calving season (71).

3.2.2 18S rDNA PCR-RFLP

This is a conserved sub-unit of the rDNA gene but contains within it a number of short polymorphic regions (Figure 7.) and it has become the "gold standard" for speciating and genotyping *Cryptosporidium* isolates.

Initial 18S rDNA nested PCR produced an amplified secondary band of 832 bp (Figure 8.), with a detection sensitivity of between 1 and 10 oocysts. Non-specific bands at the threshold of visible detection were occasionally seen. Following gel extraction and purification of the 832 bp band, these did not interfere with either the DNA analysis by RFLP or direct sequencing,

Two different endonuclease restriction enzymes were used separately in the PCR-RFLP analysis of positive isolates; *Vsp*1 resolved *C. hominis* from *C. parvum* bovine genotype and *Ssp*1 differentiated the other *Cryptosporidium* species. Endonuclease restriction digests of *C. hominis* PCR product by *Vsp*1 produced fragments of 561, 107, 85 and 82 bp but only three bands were visible on 3.5% Metaphor® agarose gel, as the two smallest bands migrated together. *C. parvum* bovine genotype *Vsp*1 endonuclease digestion produced fragments of 643, 107 and 85 bp. *Ssp*1 did not differentiate between *C. hominis* and *C. parvum* bovine genotype with both yielding fragments of 472, 238, 114 and 11 bp. The *Ssp*1 restriction enzyme did however, differentiate between the other *Cryptosporidium* species (Figure 9.).

Overlapping bi-directional direct sequencing of the 18S rDNA PCR product produced good sequence over a distance of approximately 734 bp, enabling multiple sequence alignment and phylogenetic analysis to be performed (Figures 10 and 11.).

Figure 7. *C. hominis* [GenBank AF108865] and *C. parvum* bovine genotype [GenBank AF 108864] aligned 18S rRNA gene sequences showing primary forward and reverse primers highlighted in blue, the secondary forward and reverse primers highlighted in green. The cleavage sites of the restriction enzymes, *Vsp*1 and *Ssp*1, are highlighted in yellow and red respectively.

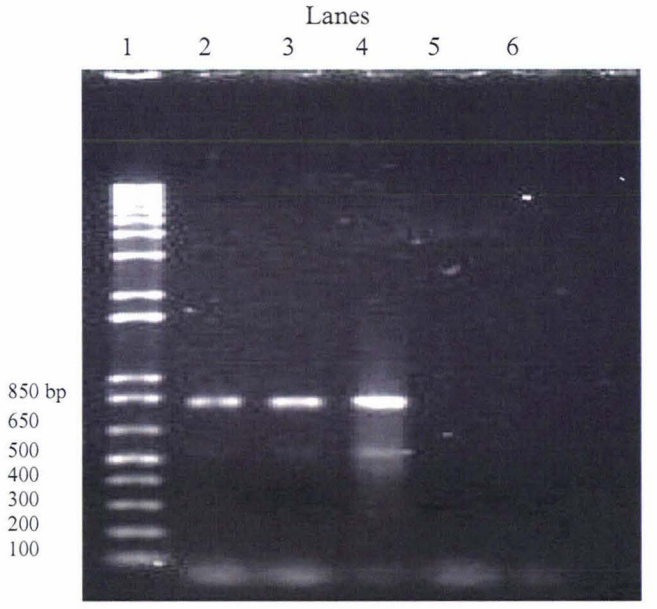


Figure 8. 18S rDNA PCR of human and bovine *Cryptosporidium* isolates. Lane 1, 1 Kb plus DNA ladder; Lanes 2 and 3, positive human samples; Lane 4, positive bovine sample; Lane 5, primary PCR negative control; Lane 6, secondary PCR negative control.

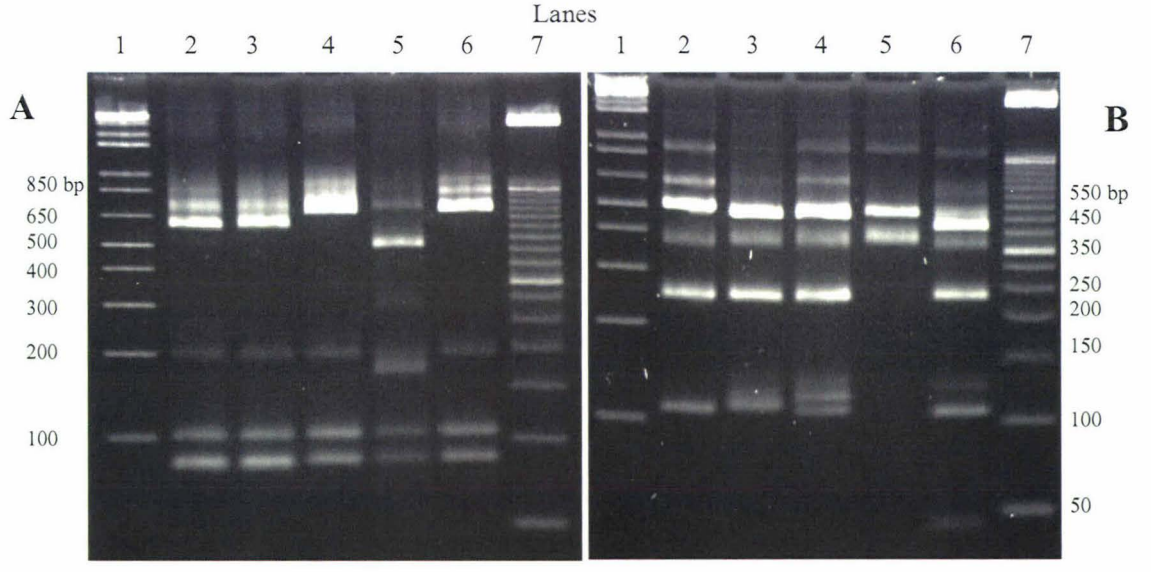


Figure 9. *Cryptosporidium* species diagnosis by *Vsp*1 (A) and *Ssp*1 (B) endonuclease restriction digestion of nested PCR 18S rDNA. Lane 1, 1 Kb plus DNA ladder; Lane 2, *C. parvum* rabbit genotype; Lane 3, *C. hominis*; Lane 4, *C. parvum* bovine genotype (from a horse); Lane 5, *C. parvum* novel genotype (from a human); Lane 6, *C. canis*; Lane 7, 50 bp DNA ladder.

Direct sequencing of the 18S rDNA PCR product was carried out on 18 isolates that had an unusual PCR-RFLP pattern or were from animals other than humans or cattle. These included three isolates of *C. canis* from dogs, *C. parvum* bovine genotype from three thoroughbred foals which were euthanased due to severe dehydration caused by diarrhoea (52), an unnamed genotype/species from a feral rabbit, two novel isolates from humans whose sequence most resembled C. *canis*, and nine other human isolates. The sequences from the two novel isolates and the rabbit have been deposited with GenBank under the accession numbers AY458612 to AY458614 respectively. For phylogenetic purposes a number of humans and bovine isolates were sequenced.

```
380                 390         400       410       420         430
.|....|....|....|....|....|....|....|....|....|....|....|....|.
Isolate 2683        TAATAATTTATATATAATATTTTATTA---ATATT--TATATAGTATTAACATAATTCAT
Isolate 2893        TAATAATTTATATATAATATTTTATTA---ATATT--TATATAGTATTAACATAATTCAT
C. canis            TAATAATTTATATATAATATTTAAC-----ATATT--TATATAATATTAACATAATTCAT
C. parvum - pig     TAATAATTTATATATAATATTT---TT--AATATT--TATATAATATTAACATAATTCAT
C. meleagridis      TAATAATTTATATATAATATTTGA-TT--AATATT--TATATAATATTAACATAATTCAT
C. parvum - bovine  TAATAATTTATATAAAATATTTTGATG--AATATT--TATATAATATTAACATAATTCAT
C. wrairi           TAATAATTTATATATAATATTTTGA-A--AATATT--TATATAATATTAACATAATTCAT
C. hominis          TAATAATTTATATAAAATATTTTGATG--AATATT--TATATAATATTAACATAATTCAT
C. parvum - rabbit  TAATAATTTATATAATATATTTTGATA--GATATT--TATATAATATTAACATAATTCAT
C. felis            TAATACCTTATATATAATATTTTTTTTTAAATATTATTATGTAAGATTAACATAATTCAT
C. baileyi          TAATA-CTTATATACAATACCAC------GGTATT--TATATAACATTAACATAATTCAC
C. muris            TG-TA--TAATCTATAATATTACTAAG---GTATA-TATTATATTATCAACATCCTTCCT
C. andersoni        TG-TA--TAATTTATAATATTACCAAG---GTAAT-TATTATATTATCAACATCCTTCCT
C. serpentis        TG-TA--TT-TTTATAATATTATTAAG---GTAAT-ATTTATAATATCAACATCCTTCCT
Clustal Consensus   *  **  *  * **  ***             **      * **  ** *****  ***

                      440         450         460         470
                     ...|....|....|....|....|....|....|....|
Isolate 2683        ATT-------ACTA---TTTT-----ATAGTATATGAA
Isolate 2893        ATT-------ACTA---TTTT-----ATAGTATATGAA
C. canis            ATT-------ACTA---TTT------ATAGTATATGAA
C. parvum - pig     ATT-------ACTATAATTTT---TATTAGTATATGAA
C. meleagridis      ATT-------ACTAAA---TT---TATTAGTATATGAA
C. parvum - bovine  ATT-------ACTATATATTT---T---AGTATATGAA
C. wrairi           ATT-------ACTATATATTT---TT--AGTATATGAA
C. hominis          ATT-------ACTATTTTTTT---TTTTAGTATATGAA
C. parvum - rabbit  ATT-------ACTATTTTTTT---T--TAGTATATGAA
C. felis            ATTTTTTAAGACTGAATTTTTAGTTTTGATAATATGAA
C. baileyi          ATT-------ACTTA--TTTA------AAGTATGTGAA
C. muris            ATT--------ATATTTCTAA------ATATATAGGAA
C. andersoni        ATT--------ATATT-CTAA------ATATATAGGAA
C. serpentis        ATT--------ATATT-TTTA------ATATATAGGAA
Clustal Consensus   ***         *                 **  ***
```

Figure 10. Multiple DNA sequence alignment, including the novel human isolates 2683 and 2893, through a polymorphic region of the *Cryptosporidium* 18S rDNA. Nucleotide position is based on the 734bp length of aligned sequence and depending on gaps in the individual sequences, actual positions are an approximation.

The 18S rDNA PCR product from the 18 isolates was sequenced and aligned over approximately 734 bp, depending on the variation in length of the individual sequences, using CLUSTAL X. A polymorphic region of the 18S rDNA is shown in Figure 10., and the entire 734 bp is shown in Appendix A (i). The 18S rDNA sequences of a number of *Cryptosporidium* species were downloaded from GenBank, aligned with PCR products from this project and a phylogenetic tree constructed to illustrate the relationship of New Zealand isolates with known species (Figure 11). Evolutionary genetic distances between the species were greater than one nucleotide change per 100bp, best illustrated by the phylogenetic tree which split the *Cryptosporidium* parasites into two monophyletic groups; the gastric *C. andersoni, C. muris* and *C. serpentis* and the intestinal remainder. A search of GenBank for an 18S rDNA sequence match to the two novel human *Cryptosporidium* isolates was unsuccessful but on the multiple species alignment they were most similar to *C. canis* isolated from the dogs. An adenine to guanine substitution at position 587 nt was the only difference between the two novel isolates. There was high conservation within New Zealand isolates of *C. hominis, C. canis* and the *C. parvum* bovine genotype sequences. This high conservation was temporal and spatial, with species sequenced in New Zealand identical to those sequenced in other countries, supporting the suggested *Cryptosporidium* clonal population structure of Awad-el-Kariem (8).

3.2.3 β-tubulin PCR-RFLP

In order to conserve expensive reagents and to use a less labour intensive PCR technique for routine genotyping of *Cryptosporidium* isolates, six other loci were investigated, two of which were unsuitable; the thrombospondin-related adhesive protein C1 (124) and an unknown repetitive DNA target (14). The β-tubulin locus detected between one and ten oocysts in two out of three attempts in a sensitivity study (Table 7.) and spans the only intron present in *Cryptosporidium.* The number of isolates that were able to be speciated/genotyped by β-tubulin PCR-RFLP totalled 295 (Table 9.), with 18S rDNA PCR-RFLP and sequencing being required on nine occasions.

On PCR-RFLP the *C. parvum* bovine genotype β-tubulin sequence had a single *Dde*1 endonuclease restriction site at position 414 which gave two fragments of 414 bp and 178 bp. The *C. hominis* remained uncut as the sequence lacked a *Dde*1 restriction site.

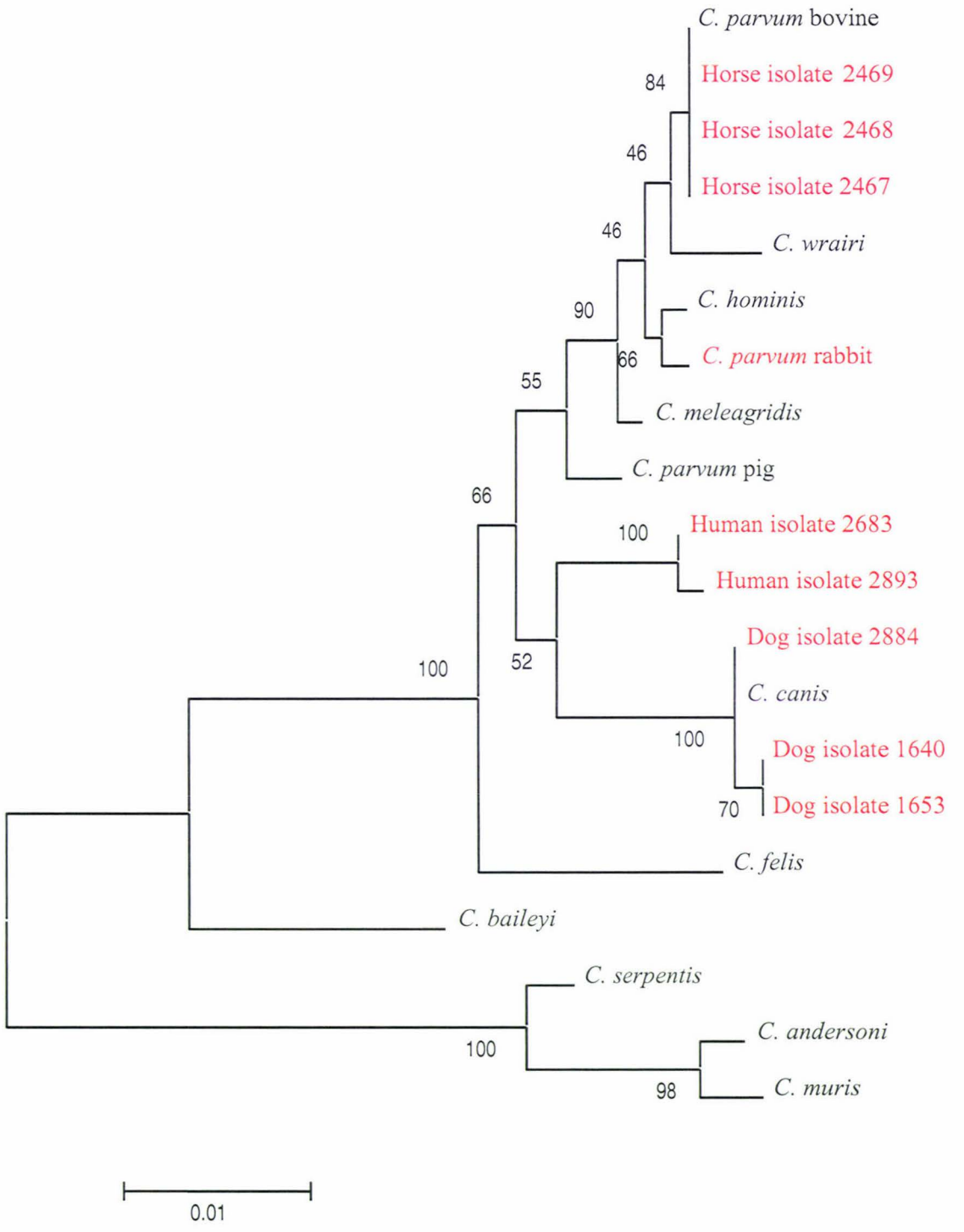


Figure 11. Neighbour-joining tree analysis of *Cryptosporidium* 18S rDNA showing the phylogenetic relationship of New Zealand isolates (red) to *Cryptosporidium* species retrieved from GenBank. Analysis was based on genetic distances calculated by the Kimura 2-parameter model with the tree was tested by 1,000 bootstrap pseudoreplications.

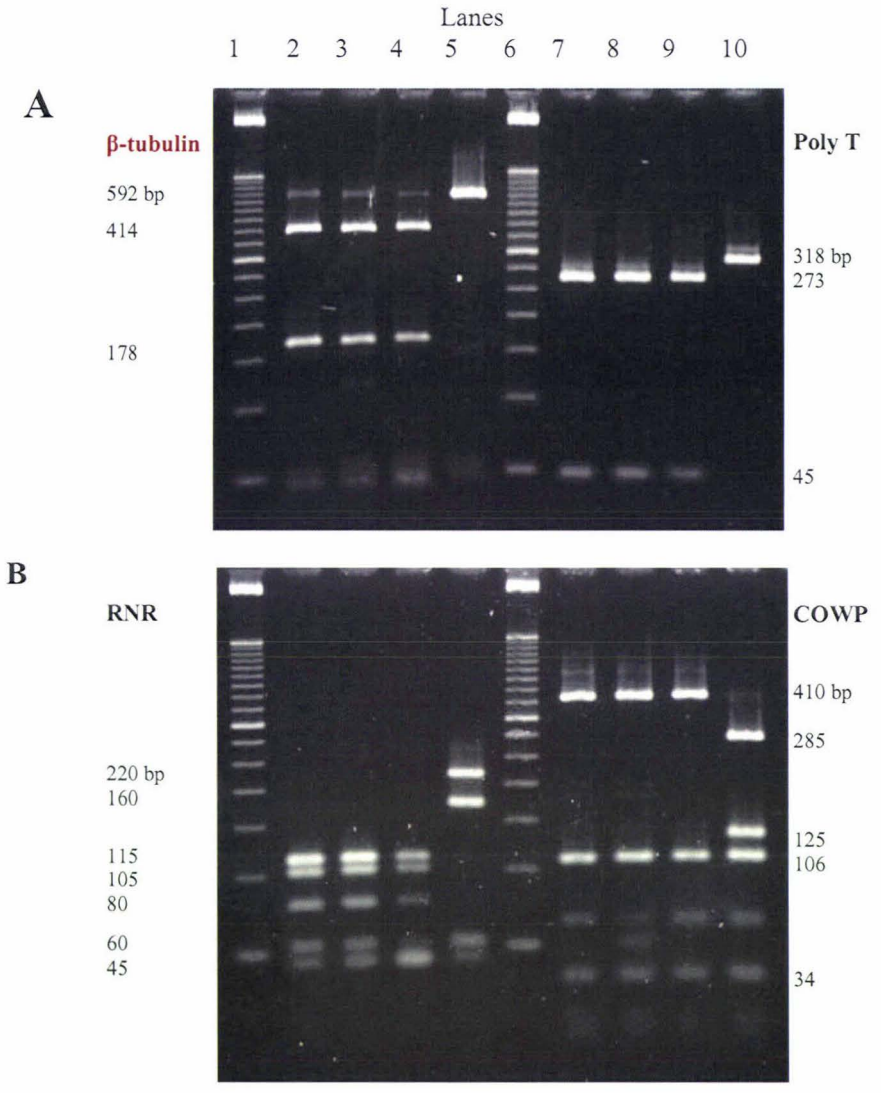
Table 9. Total number of faecal samples tested by DFA for the presence of *Cryptosporidium* oocysts from various hosts. Positive samples were speciated by either 18S rDNA PCR-RFLP and direct sequencing or β-tubulin PCR-RFLP.

Host	Total samples	C. hominis	*C. parvum* bovine	Other *Cryptosporidium*
Human	297	152	143	2 ?*C. canis*
Dog	234	0	0	3 *C. canis*
Cat	137	0	0	0
Bovine	81	0	0	0
Seagull	44	0	0	0
Lamb	34	0	0	0
Deer	11	0	0	0
Brown Teal	10	0	0	0
Rabbit	5	0	0	1
Mouse	4	0	0	0
Rat	4	0	0	0
Horse	3	0	3	0
Pig	3	0	0	0
Guinea Pig	3	0	0	0
Hedgehog	3	0	0	0
Hamster	2	0	0	0
Budgerigar	2	0	0	0
Alpaca	1	0	0	0
Total	882	152	146	6

3.2.4 Additional loci examined

Other loci examined by PCR-RFLP included, in order of sensitivity, the polythreonine repeat motif (poly T) (27), the *Cryptosporidium* oocyst wall protein gene (COWP) (125) and the ribonuclease reductase gene R1 subunit (RNR) (147). Although these loci were not used routinely they all, along with ITS-1 rDNA, 18S rDNA and β-tubulin PCR-

RFLP, speciated the rabbit *Cryptosporidium* isolate as *C. hominis* when 18S rDNA sequencing showed it to be a *C. parvum* genotype. *C. parvum* bovine genotype was diagnosed from three foals using these loci in conjunction with 18S rDNA sequencing and β-tubulin PCR-RFLP (52). PCR-RFLP fragment patterns are shown in Figure 12., and the sizes in Table 10.



Figures 12. PCR-RFLP profiles of four loci. A - the profile for β-tubulin (*Dde*1) and Poly T (*Rsa*1). B - the profile for RNR (*Tsp* 509 1) and COWP (*Rsa*1). Lanes 1 and 6, 50 bp DNA ladder; Lanes 2-4 and 7-9, *C. parvum* bovine genotype isolated from foals; Lanes 5 and 10, *C. hominis.*

Table 10. Restriction endonuclease digestion fragment sizes (bp) at six loci.

Locus	RE*	C. hominis	C. parvum *bovine*
ITS-1 rDNA	*Dra*1	349, 174, 132, 126, 104	614, 240
18S rDNA	*Vsp*1	561, 107, 85, 82	643, 107, 85
β-tubulin	*Dde*1	592	414, 178
Poly T	*Rsa*1	318	273, 45
COWP	*Rsa*1	410, 106, 34	285, 125, 106, 34
RNR**	*Tsp*509 1	220, 160, 60	115, 105, 80, 60, 45

* Restriction endonuclease.
** RNR fragment sizes are an approximation. No sequence data was available on GenBank or from G. Widmer. Attempts at sequencing PCR product were unsuccessful. Two *C. parvum* bovine genotype fragments may have migrated together.

3.3 DATA ANALYSIS

3.3.1 *Cryptosporidium* species geographic distribution

Geographic distribution of the two main *Cryptosporidium* species causing human infection reflected the land use of the regions from which the specimens were referred. Hamilton, a city servicing a large dairy intensive region, referred human samples that consisted of 40 (35%) *C. hominis*, 72 (64%) *C. parvum* bovine genotype and 1 novel *C. canis* like genotype. Human isolates from Wellington, a metropolitan city, were in direct contrast and consisted of 109 (89%) *C. hominis*, and 13 (11%) *C. parvum* bovine genotype. The remaining isolates were all from provincial New Zealand; being made up of 49 (26%) *C. hominis*, 138 (73%) *C. parvum* bovine genotype and 1 novel *C. canis* like genotype (Table 11.). All provincial centres referred more *C. parvum* bovine genotype than *C. hominis* genotype with the exception of Hawke's Bay, which referred 21 *C. hominis* genotype positive specimens during the autumn cryptosporidiosis peak and 15 *C. parvum* bovine genotype positive specimens during the spring peak. Southland, which is undergoing a boom in dairy farming, referred 35 positive specimens, 34 of which were *C. parvum* bovine genotype.

TABLE 11. Geographic distribution of *Cryptosporidium* species from New Zealand human isolates.

Locality	Number of isolates			
	C. hominis	*C. parvum* bovine genotype	Novel genotype	Total
Wellington	109 (89%)	13 (11%)		122
Hamilton	40 (35%)	72 (64%)	1 (<1%)	113
Hawkes Bay	21 (58%)	15 (42%)		36
Southland	1 (3%)	34 (97%)		35
Bay of Plenty	6 (18%)	27 (82%)		33
Hutt Valley	12 (48%)	12 (48%)	1 (4%)	25
All others*	9 (15%)	50 (85%)		59
Total	198 (47%)	223 (53%)	2 (<1%)	423

* Only localities that referred more than 20 faecal specimens are named individually.

3.3.2 *Cryptosporidium* species seasonality

Isolates from humans examined between August 2000 and June 2001 were speciated by PCR-RFLP at the ITS-1 rDNA locus and the remainder resolved by either β-tubulin PCR-RFLP or 18S rDNA sequencing. The two gaps centred around December 2000 and August 2001 in Figure 13., were due to funding constraints and not a lack of specimens.

A marked seasonal shift in transmission cycle was apparent between spring and autumn. Almost all the human infections of *Cryptosporidium* were caused by the zoonotic *C. parvum* bovine genotype during the spring, which then switched to an anthroponotic cycle of *C. hominis* infections over the autumn season (Figure 13.). This pattern has been noted previously (70, 72, 83).

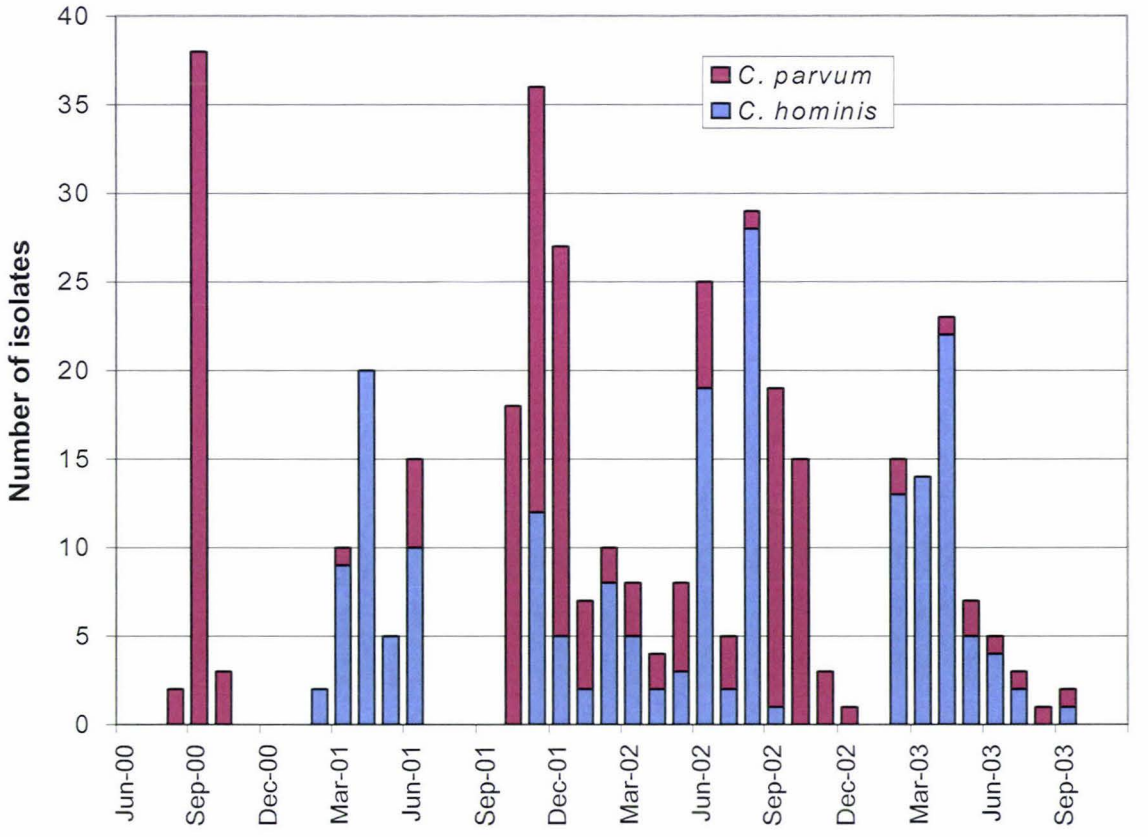


Figure 13. Seasonal changes in the transmission cycle of *Cryptosporidium sp.* isolated from humans. *C. hominis* predominates in April, May and June while *C. parvum* bovine genotype predominates in September, October, November and December.

3.4 *CRYPTOSPORIDIUM* SPECIES SUBGENOTYPING

Seventy-six human *Cryptosporidium* isolates consisting of 56 *C. hominis*, 18 *C. parvum* bovine genotype and two novel *C. canis*-like isolates were subjected to RT-PCR amplification of the two small, extrachromosomal, viral-like dsRNA found in the cytoplasm.

More isolates produced cDNA when subjected to RT-PCR of the larger of the two dsRNA molecules, with sequence data from the 259 nt fragment being obtained from 22 of the 76 isolates and a multi-sequence alignment assembled (Appendix A (ii)). Of these 5 were *C. parvum* bovine genotype and 17 *C. hominis*. All of the *C. hominis* isolates were from the Wellington region, being collected between July 2002 and May 2003.

The 5 *C. parvum* bovine genotype isolates were obtained from Bay of Plenty (2), Hamilton, Wellington and Southland.

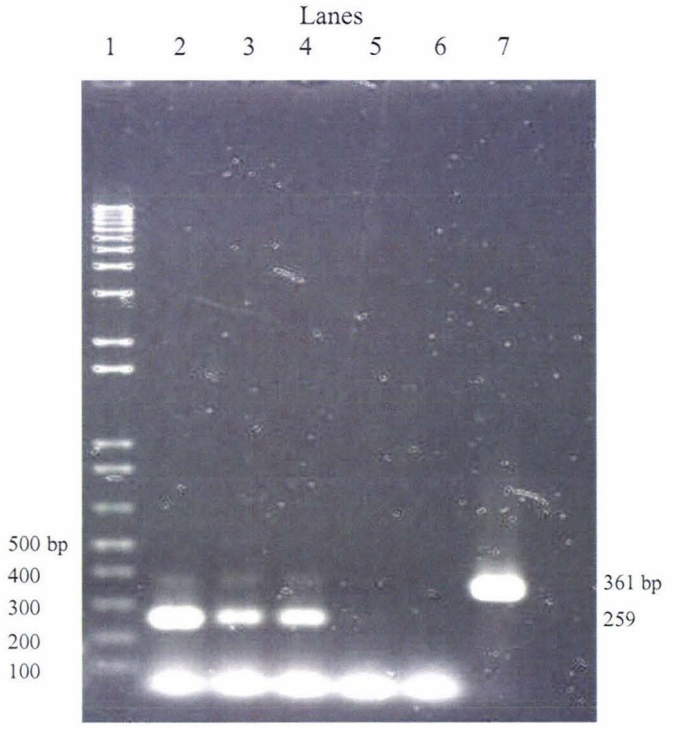


Figure 14. RT-PCR of the dsRNA from three *Cryptosporidium* isolates. Lane 1, 1 Kb plus DNA ladder; Lane 2, isolate 2491; Lane 3, isolate 2640; Lane 4, isolate 2641; Lane 5, Minus RT control; Lane 6, Minus template PCR; Lane 7, control template RNA.

Phylogenetic analysis by neighbour-joining placed all the *C. hominis* isolates into a single group and the *C. parvum* bovine genotype into 3 groups (Figure 15.). The tree was tested by bootstrap values obtained from 1000 pseudo-replications, using *C. parvum* bovine genotype BOP 2840B as an outgroup.

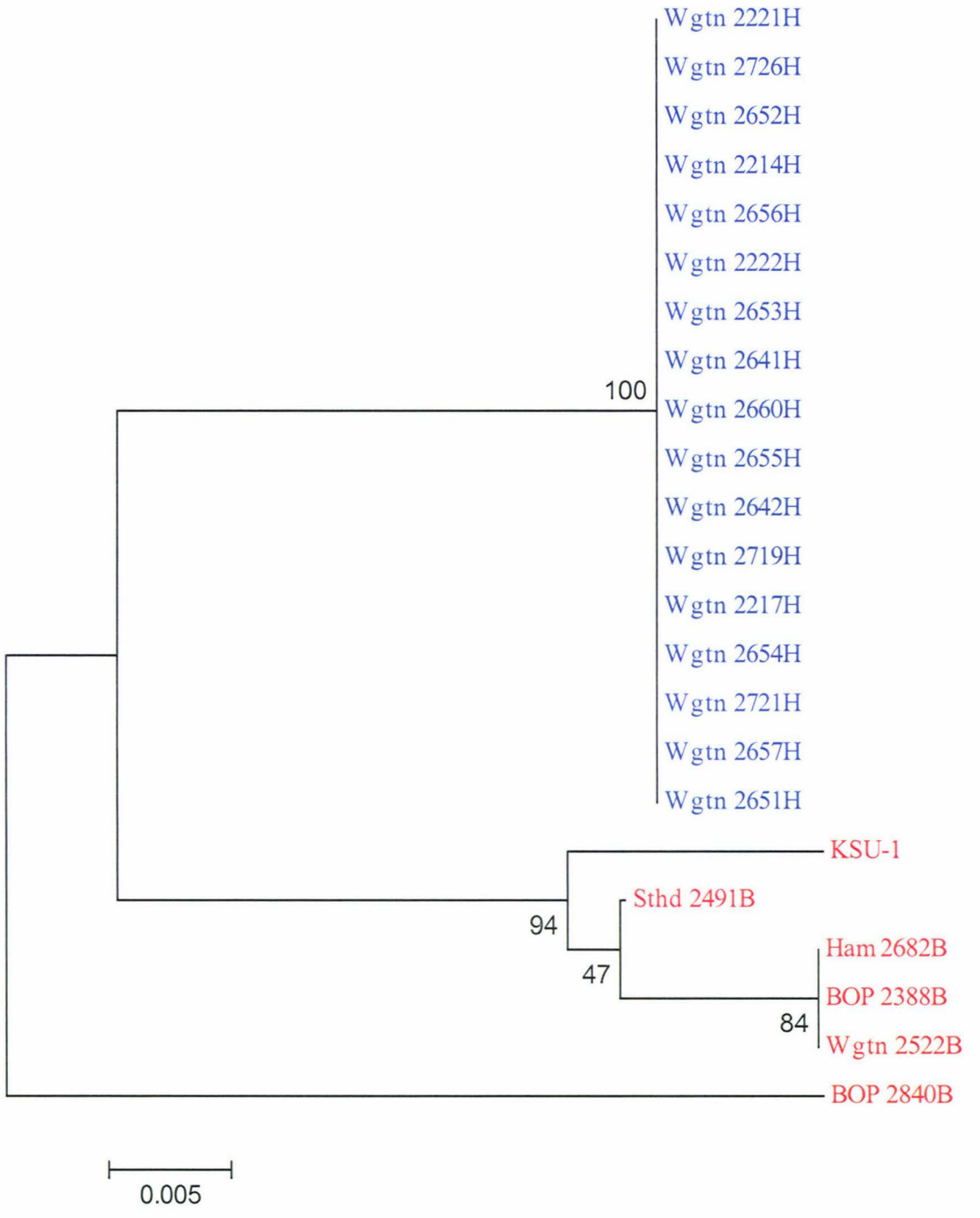


Figure 15. Phylogenetic tree inferred by neighbour joining analysis of dsRNA nucleotide sequences. The tree was tested by bootstrap values obtained from 1000 pseudo-replications, using *C. parvum* bovine genotype BOP 2840B as an outgroup.

CHAPTER 4: DISCUSSION

The primary objective of this project was to utilise the best methods available to detect, isolate and genetically characterise *Cryptosporidium* isolates from humans and animals in New Zealand. Having achieved this the secondary objective was to investigate the *Cryptosporidium* species present in New Zealand, with regard to their phylogeny, distribution and transmission cycles in the human population and assess the implications for public health. The results have similarities to those found in previous studies (83, 105).

4.1 *CRYPTOSPORIDIUM* SPECIES ISOLATED FROM ANIMALS

Animal faecal specimens were obtained from Massey University farms, Massey University Small Animal Production Unit, IVABS, AgResearch (Ruakura), AgriQuality (Palmerston North), and Gribbles Veterinary Pathology (Auckland). Of the 1613 animal specimens examined for *Cryptosporidium* only 45 (2.8%) contained oocysts and 34 (2.1%) of those came from calves. The three *Cryptosporidium* positive horses were foals and the three positive dogs were puppies, supporting the finding that this parasite is a disease of young animals. As young farm animals are predominant during the spring months and humans can be infected with *C. parvum*, it is not surprising that *C. parvum* almost exclusively causes the spring human cryptosporidiosis peak. Whether the disease is spread zoonotically initially and maintained by anthroponotic transmission may require isolates of *C. parvum* to be sub-genotyped.

During this study an isolate from the colon of a shot wild rabbit was characterised. Multi-loci PCR-RFLP was performed on the 18S rDNA, rDNA internal transcribed spacer region 1 (88), β-tubulin gene (19), *Cryptosporidium* oocyst wall protein gene (125), a poly threonine repeat motif (27) and the ribonucleotide reductase R1 sub unit locus (147) with all the results indicating *C. hominis*. The sequence however, from the 18S rDNA region of this isolate was not identical to that of *C. hominis* (Figure 10). This sequence was included in the phylogenetic analysis of strains for this study (Figure 11. *C. parvum*-rabbit). Although the sequence is unique and first published by Xiao, it is still most similar to the human genotype (118, 156).

4.2 *CRYPTOSPORIDIUM* SPECIES ISOLATED FROM HUMANS

Of the many medical diagnostic laboratories that supplied human faecal specimens containing *Cryptosporidium* oocysts on a regular basis, MedLab Hamilton - 145 and Medical Laboratory Wellington – 135, forwarded the majority. Other laboratories provided specimens on a more sporadic basis to give a total of 423. Enough specimens were received for conclusions to be drawn.

PCR-RFLP analysis of the β-tubulin gene was successful for 405 of the 423 human isolates, differentiating them into *C. hominis* and the *C. parvum* bovine genotype. Early in the study the 18S rRNA region of 20 of the 405 isolates was amplified and sequenced to check for agreement between the two methods. In every case, the results from β-tubulin PCR-RFLP and the sequenced 18S rDNA concurred. Also there was no variation within the *C. hominis* sequences or the *C. parvum* bovine genotype sequences. PCR-RFLP of the β-tubulin gene was therefore a consistent, reliable and rapid method that could be used with confidence for speciating isolates from human faecal samples.

A novel *Cryptosporidium* genotype that clustered closely to *C. canis* was detected in faecal specimens from 2 children (24 and 14 months of age) – one in Hamilton and one in Upper Hutt (close to Wellington). Both were detected by nested 18S rDNA PCR and then only a small amount of PCR product was produced, indicating a light oocyst load with a genotype that may have accidentally infected humans.

The overall numeric distribution of the two *Cryptosporidium* species was almost equally divided; *C. hominis* 198 (46.8%) and *C. parvum* bovine genotype 223 (52.7%), with the *C. canis* like genotype numbering two (0.5%). This numeric distribution of *Cryptosporidium* species gives an incomplete picture, for when the provincial regions are compared to metropolitan Wellington there is a marked difference. Wellington had 109 (89%) *C. hominis* and 13 (11%) *C. parvum* bovine genotype, whereas the provincial regions were made up of 89 (30%) *C. hominis* and 210 (70%) *C. parvum* bovine genotype with the *C. canis* like genotype numbering two. New Zealand's temperate climate allows livestock to remain outdoors on pasture year round with the Hamilton region capable of having two calving seasons, resulting in a potential for an increase in the environmental load of oocysts. The spreading of ponded milking shed

washings and meat processing plant effluent as fertiliser onto land, plus the frequent mild rainfall also disperses the oocysts over pastures and ultimately into rivers and streams. An earlier study found *Cryptosporidium* oocysts to be present in 12.6% of environmental and treated waters tested from throughout New Zealand (16). With today's more efficient sampling, recovery and detection procedures this number would be expected to be higher. People living in Wellington have less opportunity for contact with farm animals but would have more chance of being involved in outbreak situations, be it person to person, water or food-borne. The lack of access to the countryside rather than the lack of animals has previously been suggested as an explanation for a decline in cryptosporidiosis caused by *C. parvum* bovine genotype (58).

Cryptosporidiosis has been a notifiable disease in New Zealand since July 1996 with case numbers rising each year. New Zealand's infection rate (cases per 100,000) for the years 2001 and 2002 were 32.3 and 26.1 respectively, however both Hamilton (61.2 and 40) and Wellington (37.4 and 61) were above the national rate (11). Although the national infection rate fell during 2002, Wellington's infection rate increased to 61 as it experienced 2 swimming pool outbreaks resulting in 72 cases of cryptosporidiosis with *C. hominis.* Notified cases have a distinct seasonal pattern, usually showing two clear peaks during spring and autumn (11) (Figure 16.). The autumns of 2000, 2002 and 2004 however, did not show peaks, which might be attributed to a lack of outbreaks from swimming pools, play centres or food and drink. Spring is the main calving and lambing season and as *Cryptosporidium* is a disease of young animals, this is the time of year when there is a high environmental load with *C. parvum* bovine genotype. *C. parvum* bovine genotype was responsible for almost all the spring cryptosporidiosis cases before a complete change of transmission cycle saw *C. hominis* dominant in the autumn. This seasonality was reflected in the Hamilton specimens with *C. hominis* prevalent in the autumn and *C. parvum* bovine genotype in the spring. Wellington referred fewer spring positive faecal specimens, which may reflect the lack of access to young infected animals by its population. Figure 13 shows this seasonal shift in transmission cycles. As humans are usually infected only by either *C. hominis* or *C. parvum* bovine genotype, more discriminating methods other than PCR-RFLP and 18S rDNA sequencing are needed to investigate the source of outbreaks. Molecular techniques capable of detecting

intra and inter *Cryptosporidium* species/genotype genetic polymorphisms are required and are now being investigated (5, 18, 42, 47, 64, 73, 78, 79, 127, 149).

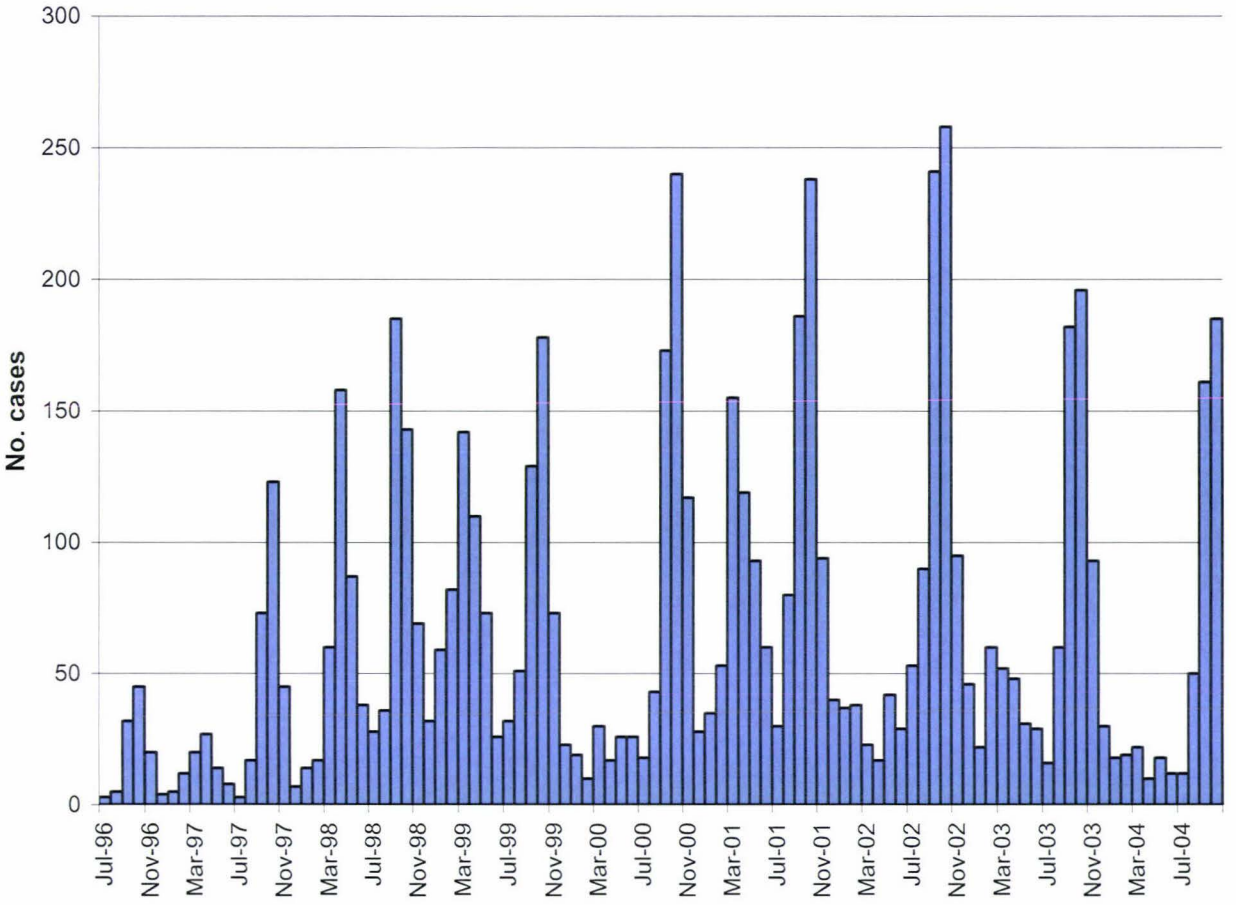


Figure 16. National monthly numbers of cryptosporidiosis cases notified to the New Zealand Ministry of Health since July 1996 when the disease became notifiable.

New Zealand's climate, animal husbandry and farming practises are ideally suited to the spread and survival of *Cryptosporidium* oocysts. Fencing off rivers and streams or planting riparian buffer zones along stream banks could restrict the access of livestock and their excreta to natural waterways. Currently the government is considering submissions on public access across private land to natural waterways, with the fencing of buffer zones being one possibility. Fonterra, New Zealand's largest dairy company which is a co-operative owned by farmers, is actively encouraging its shareholders to fence off esplanade strips along natural waterways to reduce the concept of "dirty dairying". Planted buffer zones can remove up to 99.9% of *Cryptosporidium* oocysts

during low to moderate rainfall, depending on soil type, slope and distance to water (7). Effective wastewater management of effluent from farms, meat processing plants, settlements, towns and cities could interrupt the transmission cycle along with keeping the water reticulation of toddlers' public swimming pools separate from the main swimming pool. A combination of education, land use regulation and perhaps economic incentives may be required to enable the success of the above suggestions. Vigilance with public health education programmes and strict adherence to the New Zealand Drinking Water Standards would also help to lower the infection rate.

4.3 VIRUS-LIKE, SMALL dsRNA SUB-GENOTYPING

Cryptosporidiosis in New Zealand has two peaks per year (Figure 16.), with a change in transmission cycle from *C. hominis* during autumn to *C. parvum* bovine genotype during spring (Figure 15.). Between the two peaks the background level of cryptosporidiosis cases drops to low levels (11). Epidemiological investigation of these peaks requires a technique that goes beyond the species level and examines intraspecies heterogeneity, as humans are usually only infected by either *C. hominis* or *C. parvum* bovine genotype. This study of a RT-PCR technique targeting the virus-like, small (259 nt), linear, extrachromosomal dsRNA found in *Cryptosporidium* sporozoites was performed to see if it was capable of being used for genetic epidemiological purposes to allow targeted intervention to block transmission routes.

For the period of this study the only *C. hominis* isolates from which sufficient RNA for analysis could be extracted came from the Wellington region and the majority were collected during the autumn. All belonged to the same *C. hominis* sub-genotype. Two outbreaks occurred in Wellington during the autumn of 2002 and at least one in the autumn of 2003. All outbreaks were associated with swimming pools and our findings indicate that there was only the one sub-genotype of *C. hominis* circulating in Wellington at the time.

More diversity was seen with the *C. parvum* bovine genotype although only five isolates were able to be sub-typed. Specimens able to be sequenced came from Bay of Plenty (2), Hamilton (1), Wellington (1) and Southland (1). These five were divided into three sub-genotypes with one group consisting of isolates from three regions

showing that a sub-genotype of *C. parvum* bovine genotype can circulate in several regions simultaneously. Conversely the two Bay of Plenty isolates sub-genotyped into different groups showing that more than one variant of *C. parvum* bovine genotype can circulate in a region simultaneously.

Of the 76 *Cryptosporidium* isolates processed by RT-PCR only 22 gave sufficient product to sequence and hence sub-genotype. This was the disadvantage of this method – the labour input exceeded the amount of useful information gained. RNA is a difficult nucleic acid to work with but previous workers did not have the problems we encountered and obtained data from every isolate (64, 65, 152). The extended time period between the specimens being collected and our examination of them could explain this. It was up to eight weeks before some specimens could be examined and endogenous RNases may have degraded the RNA during the interval.

Subgenotyping of *Cryptosporidium* species is required to study the complex epidemiology of this parasite. This is of particular importance in outbreak situations as shown by the Wellington isolates in this study. As this method was not suitable to be carried out by a distant reference laboratory a technique utilising the more stable DNA may be what is required. DNA sites that have been examined previously include mini and micro-satellites (18, 22, 78), the 70 kDa heat shock protein (HSP70) (130), the thrombospondin-related protein C2 (130) and the 60 kDa glycoprotein (GP60) genes (5, 109, 130).

4.4 SUMMARY AND FUTURE DIRECTIONS

Of the 1613 animal faecal specimens examined for *Cryptosporidium* oocysts only 45 (2.8%) contained oocysts and 34 (2.1%) of those were from calves. As the disease is one of young animals, environmental oocyst load is greatest during the spring when separation of farm animals from natural waterways would reduce the potential for human waterborne infection. The collection of specimens from animals showing symptoms of cryptosporidiosis may have uncovered a larger number and variety of host adapted species/genotypes.

Immunocompetent humans can be infected by the either *C. hominis* or *C. parvum* bovine genotype, although *C. meleagridis*, *C. muris, C, suis, C. felis* and *C. canis* infections have been reported (25, 70, 104, 105, 119, 157). New Zealand wide numeric distribution of the two species in the 423 *Cryptosporidium* positive humans, was approximately equal – *C. hominis* 198 (46.8%) and *C. parvum* bovine genotype 223 (52.7%). The two species were, however distributed along rural/urban lines: rural - *C. parvum* bovine genotype 210 (70%), *C. hominis* 89 (30%); urban – *C. hominis* 109 (89%), *C. parvum* bovine genotype 13 (11%). Both species had a distinct seasonal transmission cycle with a zoonotic cycle in the spring followed by an anthroponotic cycle in the autumn.

Targeted intervention to disrupt the transmission cycle will require a thorough analysis of *Cryptosporidium* population genetics. Investigation of the small, extachromosomal, virus-like dsRNA yielded some useful information but clearly a more effective approach is required. Studies by Sulaiman *et al.* (2001) and Mallon *et al.* (2003) using multilocus genotyping (78, 79, 130) have produced useful information on the population structure of this parasite. The Scottish study analysed linkage disequilibrium between pairs of alleles and their genetic distances at seven different loci in the 38 sub-genotypes of *C. hominis* and *C. parvum* bovine genotype they found, resulting in a finding of four genetically isolated *Cryptosporidium* populations (78). Their results supported a clonal population structure for *C. hominis* but found a panmictic population structure for the *C. parvum* bovine genotype isolated from both cattle and humans. A human host adapted *C. parvum* bovine genotype sub-genotype was also found indicating that not all *C. parvum* bovine genotype infections are transmitted zoonotically. A human host adapted sub-genotype of *C. parvum* bovine genotype has been found previously (5, 151).

The population genetic structure of New Zealand *Cryptosporidium* isolates, both animal and human, needs to be determined to understand the epidemiology and transmission of this parasite (154). This may require a multilocus sequencing approach but now that the genomic sequences of *C. hominis* and *C. parvum* bovine genotype have been completed a second generation of high-resolution molecular sub-genotyping tools are becoming available (1, 158).

APPENDICES

APPENDIX A: ABBREVIATIONS

Abbreviations commonly used throughout this thesis.

°C	Degrees Celsius
AIDS	Autoimmune deficiency syndrome
AT	Adenine/thiamine
BOP	Bay of Plenty
bp	Base pair
BSA	Bovine serum albumin
cm	Centimetre
COWP	*Cryptosporidium* outer wall protein
DAPI	6-diamidino-2-phenylindole
DFA	Direct fluorescent antibody
DHFR	Dihydrofolate reductase-thymidylate synthase
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetra-acetic acid
EIA	Enzyme immunoassay
ESR	Environmental Science and Research Ltd.
FISH	Fluorescent in situ hybridisation
FITC	Fluorescein-isothiocyanate
g	Grams or gravity
GP60	Glycoprotein 60
Ham	Hamilton
HCl	Hydrochloric acid
Hsp70	Heat shock protein 70
IMS	Immunomagnetic separation
ITS	Internal transcribed spacer region
IVABS	Institute of Veterinary, Animal and Biomedical Sciences

Kbp	Kilo base pairs
kDa	KiloDalton
KSU	Kansas State University
L	Litre
M	Molar
Mbp	Mega base pairs
mg	Milligram
min	Minute
mL	Milli-litre
mM	Millimolar
mRNA	Messenger RNA
nm	Nanometre
nt	Nucleotide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propridium iodide
Poly T	Polythreonine
PRU	Protozoa Research Unit
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal DNA
RFLP	Restriction length polymorphism
RNA	Ribonucleic acid
RNR	Ribonucleotide reductase
RPM	Revolutions per minute
RT-PCR	Reverse transcriptase PCR
s	Second
S	Svedberg
SDGC	Sucrose density gradient centrifugation
SNP	Single nucleotide polymorphism
Sthd	Southland
TE	Tris-ethylenediaminetetra-acetic acid
TRAP	Thrombospondin-related adhesive protein
USEPA	United States Environment Protection Agency

V/cm	Volts per centimetre
w/v	Weight to volume
Wgtn	Wellington
ZN	Ziehl-Nelseen
β-tubulin	Beta-tubulin
μg	Microgram
μL	Microlitres
μm	Micron
μM	Micromolar

APPENDIX B: SEQUENCE DATA

The sequence data used to produce the 18S rDNA and extrachromosomal, viral-like, dsRNA phylogenetic trees are included below.

Appendix B (i)

Multiple 18S rDNA sequence alignment, including the novel human isolates 2683 and 2893, three *C. canis* dog isolates, a feral rabbit and three *C. parvum* bovine genotypes isolated from foals.

```
#MEGA
!Title : 18S-msf.;
!Format
   DataType=Nucleotide CodeTable=Standard
   NSeqs=20 NSites=734
   Identical=. Missing=? Indel=-;

!Domain=Data property=Coding CodonStart=1;
#C. hominis        ATC ACA --- -AT T-- AAT GTG ACA TAT CAT TCA AGT TTC TGA CCT ATC
#C. parvum rabbit  ... ... --- -.. .-- ... ... ... ... ... ... ... ... ... ...
#Isolate 2683      ... ... --- -T. .A- T.. ... ... ... ... ... ... ... ... ...
#Isolate 2893      ... ... --- -T. .A- T.. ... ... ... ... ... ... ... ... ...
#Dog 1640          ... ... --- -T. .-- T.. ... ... .C. ... ... ... ... ... ...
#Dog 1653          ... ... --- -T. .-- T.. ... ... .C. ... ... ... ... ... ...
#Dog 2884          ... ... --- -T. .-- T.. ... ... ... ... ... ... ... ... ...
#C. canis          ... ... --- -T. .-- T.. ... ... ... ... ... ... ... ... ...
#C. parvum pig     ... ... --- -T. .TT ... ... ... ... ... ... ... ... ... ...
#C. meleagridis    ... ... --- -.. .T- -.. ... ... ... ... ... ... ... ... ...
#C. parvum bovine  ... ... --- -T. A-- ... ... ... ... ... ... ... ... ... ...
#Horse 2469        ... ... --- -T. A-- ... ... ... ... ... ... ... ... ... ...
#Horse 2468        ... ... --- -T. A-- ... ... ... ... ... ... ... ... ... ...
#Horse 2467        ... ... --- -T. A-- ... ... ... ... ... ... ... ... ... ...
#C. wrairi         ... ... --- -TA A-- .T. ... ... ... ... ... ... ... ... ...
#C. felis          ... ... ATA AT. .AT TT. ... ... ... ... ... ... ... ... ...
#C. baileyi        ... ... --- -T. .-- -.. ... ... ... ... ... ... ... ... ...
#C. muris          ... G.. --- -TC .CT G.. .C. ... ... ... ... ... ... ... ...
#C. andersoni      ... G.. --- -TC .CT G.. .C. ... ... ... ... ... ... ... ...
#C. serpentis      ... G.. --- -TC .CT G.. .C. ... ... ... ... ... ... ... ...
```

#*C. hominis*	AGC	TTT	AGA	CGG	TAG	GGT	ATT	GGC	CTA	CCG	TGG	CAA	TGA	CGG	GTA	ACG	
#*C. parvum* rabbit	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Isolate 2683	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Isolate 2893	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Dog 1640	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Dog 1653	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Dog 2884	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. canis*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. parvum* pig	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. meleagridis*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. parvum* bovine	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Horse 2469	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Horse 2468	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Horse 2467	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. wrairi*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. felis*	...	...	...	...	...	...	...	...	...	...	...	.T.	...	...	...	...	
#*C. baileyi*	...	...	...	...	...	...	...	...	...	...	...	.T.	...	...	...	...	
#*C. muris*	...	...	...	...	...	...	...	...	...	...	...	.T.	...	...	...	...	
#*C. andersoni*	...	...	...	...	...	...	...	...	...	...	...	.T.	...	...	...	...	
#*C. serpentis*	...	...	...	...	...	...	...	...	...	...	...	.T.	...	...	...	...	

#*C. hominis*	GGG	AAT	TAG	GGT	TCG	ATT	CCG	GAG	AGG	GAG	CCT	GAG	AAA	CGG	CTA	CCA	
#*C. parvum* rabbit	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Isolate 2683	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Isolate 2893	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Dog 1640	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Dog 1653	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Dog 2884	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. canis*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. parvum* pig	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. meleagridis*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. parvum* bovine	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Horse 2469	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Horse 2468	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Horse 2467	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. wrairi*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. felis*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. baileyi*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. muris*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. andersoni*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. serpentis*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	

#*C. hominis*	CAT	CTA	AGG	AAG	GCA	GCA	GGC	GCG	CAA	ATT	ACC	CAA	TCC	TAA	TAC	AGG	
#*C. parvum* rabbit	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Isolate 2683	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Isolate 2893	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Dog 1640	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Dog 1653	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Dog 2884	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. canis*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. parvum* pig	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. meleagridis*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. parvum* bovine	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Horse 2469	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Horse 2468	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#Horse 2467	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. wrairi*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. felis*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
#*C. baileyi*	...	...	...	...	...	...	...	...	...	...	...	...	...	.G.	C..	...	
#*C. muris*	...	...	...	...	...	...	...	...	...	...	...	...	...	.G.	C..	...	
#*C. andersoni*	...	...	...	...	...	...	...	...	...	...	...	...	...	.G.	C..	...	
#*C. serpentis*	...	...	...	...	...	...	...	...	...	...	...	...	...	.G.	C..	...	

```
#C. hominis       GAG GTA GTG ACA AGA AAT AAC AAT ACA GGA CTT TTT --G GTT TTG TAA
#C. parvum rabbit ... ... ... ... ... ... ... ... ... ... ... ... --. ... ... ...
#Isolate 2683     ... ... ... ... ... ... ... ... ... ... ... ... .AA ATA ... ... ...
#Isolate 2893     ... ... ... ... ... ... ... ... ... ... ... ... .AA ATA ... ... ...
#Dog 1640         ... ... ... ... ... ... ... ... ... ... ... ... .AA -CA ... ... ...
#Dog 1653         ... ... ... ... ... ... ... ... ... ... ... ... .AA -CA ... ... ...
#Dog 2884         ... ... ... ... ... ... ... ... ... ... ... ... .AA -CA ... ... ...
#C. canis         ... ... ... ... ... ... ... ... ... ... ... ... .AA -CA ... ... ...
#C. parvum pig    ... ... ... ... ... ... ... ... ... ... ... ... ... T-A ... ... ...
#C. meleagridis   ... ... ... ... ... ... ... ... ... ... ... ... ... --. ... ... ...
#C. parvum bovine ... ... ... ... ... ... ... ... ... ... ... ... ... --. ... ... ...
#Horse 2469       ... ... ... ... ... ... ... ... ... ... ... ... ... --. ... ... ...
#Horse 2468       ... ... ... ... ... ... ... ... ... ... ... ... ... --. ... ... ...
#Horse 2467       ... ... ... ... ... ... ... ... ... ... ... ... ... --. ... ... ...
#C. wrairi        ... ... ... ... ... ... ... ... ... ... ... ... ... --. ... ... ...
#C. felis         ... ... ... ... ... ... ... ... ... ... ... ... .AC --. ... ... ...
#C. baileyi       ... ... ... ... ... ... ... ... ... ... ..G .C. AAC --. ..C ... ...
#C. muris         ... ... ... ... ... ... ... ... ... ... ..G .C. AAC --. ..C ... ...
#C. andersoni     ... ... ... ... ... ... ... ... ... ... ..G .C. AAC --. ..C ... ...
#C. serpentis     ... ... ... ... ... ... ... ... ... ... ..G .C. AAC --. ..C ... ...
```

```
#C. hominis       TTG GAA TGA GTT AAG TAT AAA CCC CTT TAC AAG TAT CAA TTG GAG GGC
#C. parvum rabbit ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Isolate 2683     ... ... ... ... G.. ... ... ... ... ... ... ... ... ... ... ...
#Isolate 2893     ... ... ... ... G.. ... ... ... ... ... ... ... ... ... ... ...
#Dog 1640         ... ... ... ... G.. ... ... ... ... ... ... ... ... ... ... ...
#Dog 1653         ... ... ... ... G.. ... ... ... ... ... ... ... ... ... ... ...
#Dog 2884         ... ... ... ... G.. ... ... ... ... ... ... ... ... ... ... ...
#C. canis         ... ... ... ... G.. ... ... ... ... ... ... ... ... ... ... ...
#C. parvum pig    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. meleagridis   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. parvum bovine ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Horse 2469       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Horse 2468       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Horse 2467       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. wrairi        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. felis         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. baileyi       ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ... ...
#C. muris         ... ... ... ..G ... ... ... ... ... ... ... G.. ... ... ... ...
#C. andersoni     ... ... ... ..G ... ... ... ... ... ... ... G.. ... ... ... ...
#C. serpentis     ... ... ... ..G ... ... ... ... ... ... ... ... ... ... ... ...
```

```
#C. hominis       AAG TCT GGT GCC AGC AGC CGC GGT AAT TCC AGC TCC AAT AGC GTA TAT
#C. parvum rabbit ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Isolate 2683     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Isolate 2893     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Dog 1640         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Dog 1653         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Dog 2884         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. canis         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. parvum pig    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. meleagridis   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. parvum bovine ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Horse 2469       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Horse 2468       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Horse 2467       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. wrairi        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. felis         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. baileyi       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. muris         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. andersoni     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. serpentis     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
```

#*C. hominis*	TAA	AGT	TGT	TGC	AGT	TAA	AAA	GCT	CGT	AGT	TGG	ATT	TCT	GTT	AAT	AAT
#*C. parvum* rabbit	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#Isolate 2683	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#Isolate 2893	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#Dog 1640	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#Dog 1653	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#Dog 2884	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#*C. canis*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#*C. parvum* pig	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#*C. meleagridis*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#*C. parvum* bovine	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#Horse 2469	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#Horse 2468	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#Horse 2467	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#*C. wrairi*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
#*C. felis*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.CC
#*C. baileyi*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	.-C
#*C. muris*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	G-.	.--
#*C. andersoni*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	G-.	.--
#*C. serpentis*	...	...	...	...	...	...	...	...	...	...	...	...	...	...	G-.	.--

#*C. hominis*	TTA	TAT	AAA	ATA	TTT	TGA	TG-	-AA	TAT	T--	TAT	ATA	ATA	TTA	ACA	TAA
#*C. parvum* rabbit	...	...	..T	...	...	...	.A-	-G.	...	.--	...	...	...	...	...	...
#Isolate 2683	...	...	.T.	...	...	.AT	.A-	--.	...	.--	...	...	G..	...	...	...
#Isolate 2893	...	...	.T.	...	...	.AT	.A-	--.	...	.--	...	...	G..	...	...	...
#Dog 1640	...	...	.T.	...	...	AAC	---	--.	...	.--	...	...	...	...	...	...
#Dog 1653	...	...	.T.	...	...	AAC	---	--.	...	.--	...	...	...	...	...	...
#Dog 2884	...	...	.T.	...	...	AAC	---	--.	...	.--	...	...	...	...	...	...
#*C. canis*	...	...	.T.	...	...	AAC	---	--.	...	.--	...	...	...	...	...	...
#*C. parvum* pig	...	...	.T.	...	...	---	.T-	-..	...	.--	...	...	...	...	...	...
#*C. meleagridis*	...	...	.T.	...	...	GA-	.T-	-..	...	.--	...	...	...	...	...	...
#*C. parvum* bovine	...	...	...	...	...	...	..-	-..	...	.--	...	...	...	...	...	...
#Horse 2469	...	...	...	...	...	...	..-	-..	...	.--	...	...	...	...	...	...
#Horse 2468	...	...	...	...	...	...	..-	-..	...	.--	...	...	...	...	...	...
#Horse 2467	...	...	...	...	...	...	..-	-..	...	.--	...	...	...	...	...	...
#*C. wrairi*	...	...	.T.	...	...	...	-A-	-..	...	.--	...	...	...	...	...	...
#*C. felis*	...	...	.T.	...	...	.TT	.TT	A..	...	.AT	...	G..	.G.	...	...	...
#*C. baileyi*	...	...	.C.	...	CCA	C--	---	-GG	...	.--	...	...	.C.	...	...	...
#*C. muris*	.A.	.C.	.T.	...	..A	CT.	A.-	--G	...	A-T	AT.	...	T..	.C.	...	.CC
#*C. andersoni*	.A.	.T.	.T.	...	..A	CC.	A.-	--G	..A	.-T	AT.	...	T..	.C.	...	.CC
#*C. serpentis*	..-	.T.	.T.	...	..A	.T.	A.-	--G	..A	.-A	.T.	...	...	.C.	...	.CC

#*C. hominis*	TTC	ATA	TT-	---	---	ACT	ATT	TTT	TT-	--T	TTT	AGT	ATA	TGA	AAT	TTT
#*C. parvum* rabbit	...	...	..-	---	---	...	...	...	..-	--.	--.	...	...	...	...	...
#Isolate 2683	...	...	..-	---	---	...	.--	-..	..-	---	-A.	...	...	...	...	...
#Isolate 2893	...	...	..-	---	---	...	.--	-..	..-	---	-A.	...	...	...	...	...
#Dog 1640	...	...	..-	---	---	...	.--	-..	.--	---	-A.	...	...	...	..C	...
#Dog 1653	...	...	..-	---	---	...	.--	-..	.--	---	-A.	...	...	...	..C	...
#Dog 2884	...	...	..-	---	---	...	.--	-..	.--	---	-A.	...	...	...	..C	...
#*C. canis*	...	...	..-	---	---	...	.--	-..	.--	---	-A.	...	...	...	..C	...
#*C. parvum* pig	...	...	..-	---	---	...	..A	A..	..-	--.	A..	...	...	...	...	...
#*C. meleagridis*	...	...	..-	---	---	...	.AA	---	..-	--.	A..	...	...	...	...	...
#*C. parvum* bovine	...	...	..-	---	---	...	..A	.A.	..-	--.	---	...	...	...	...	...
#Horse 2469	...	...	..-	---	---	...	..A	.A.	..-	--.	---	...	...	...	...	...
#Horse 2468	...	...	..-	---	---	...	..A	.A.	..-	--.	---	...	...	...	...	...
#Horse 2467	...	...	..-	---	---	...	..A	.A.	..-	--.	---	...	...	...	...	...
#*C. wrairi*	...	...	..-	---	---	...	..A	.A.	..-	--.	.--	...	...	...	...	...
#*C. felis*	...	...	..T	TTT	AAG	...	GAA	...	..A	GT.	..G	.TA	...	...	...	...
#*C. baileyi*	...	.C.	..-	---	---	...	TA-	-..	.A-	---	--A	...	..G	...	..C	...
#*C. muris*	...	C..	..-	---	---	-A.	...	.C.	AA-	---	--A	TA.	...	G..	..C	...
#*C. andersoni*	...	C..	..-	---	---	-A.	...	-C.	AA-	---	--A	TA.	...	G..	...	...
#*C. serpentis*	...	C..	..-	---	---	-A.	...	-..	.A-	---	--A	TA.	...	G..	...	...

```
#C. hominis       ACT TTG AGA AAA TTA GAG TGC TTA AAG CAG GCA TAT GCC TTG AAT ACT
#C. parvum rabbit ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Isolate 2683     ... ... ... ... ... ... ... ... ... ... ... .TA ... ... ... ...
#Isolate 2893     ... ... ... ... ... ... ... ... ... ... ... .TA ... ... ... ...
#Dog 1640         ... ... ... ... ... ... ... ... ... ... ..T .T. ... ... ... ...
#Dog 1653         ... ... ... ... ... ... ... ... ... ... ..T .T. ... ... ... ...
#Dog 2884         ... ... ... ... ... ... ... ... ... ... ..T .T. ... ... ... ...
#C. canis         ... ... ... ... ... ... ... ... ... ... ..T .T. ... ... ... ...
#C. parvum pig    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. meleagridis   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. parvum bovine ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Horse 2469       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Horse 2468       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Horse 2467       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. wrairi        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. felis         ... ... ... ... ... ... ... ... ... ... ..T .T. ... ... ... ...
#C. baileyi       ... ... ... ... ... ... ... ... ... ... ..T AT. ... ... ... ...
#C. muris         ... ... ... ... ... ... ... ... ... ... ... AC. ... ... ... ...
#C. andersoni     ... ... ... ... ... ... ... ... ... ... ... AC. ... ... ... ...
#C. serpentis     ... ... ... ... ... ... ... ... ... ... ... AC. ... ... ... ...
```

```
#C. hominis       CCA GCA TGG AAT AAT A-T TAA AGA TTT TTA TCT TTT TT- -AT TGG TTC
#C. parvum rabbit ... ... ... ... ... .-. ... ... ... ... ... ..C ..- -.. ... ...
#Isolate 2683     ... ... ... ... ... .-. A.. ... ... ... ... ... ..- -.. ... ...
#Isolate 2893     ... ... ... ... ... .-. A.. ... ... ... ... ... ..- -.. ... ...
#Dog 1640         AG. ... ... ... ... .-. ... ... ... ... ... ..C ..- -.. ... ...
#Dog 1653         AG. ... ... ... ... .-. ... ... ... ... ... ..C ..- -.. ... ...
#Dog 2884         AG. ... ... ... ... .-. ... ... ... ... ... ..C ..- -.. ... ...
#C. canis         AG. ... ... ... ... .-. ... ... ... ... ... ..C ..- -.. ... ...
#C. parvum pig    ... ... ... ... ... .-. A.. ... ... ... ... ... ..- -.. ... ...
#C. meleagridis   ... ... ... ... ... .-. ... ... ... ... ... ..C ..- -.. ... ...
#C. parvum bovine ... ... ... ... ... .-. ... ... ... ... ... ..C ..- -.. ... ...
#Horse 2469       ... ... ... ... ... .-. ... ... ... ... ... ..C ..- -.. ... ...
#Horse 2468       ... ... ... ... ... .-. ... ... ... ... ... ..C ..- -.. ... ...
#Horse 2467       ... ... ... ... ... .-. ... ... ... ... ... ..C ..- -.. ... ...
#C. wrairi        ... ... ... ... ... .-. ... ... ... ... ... ..C ..- -.. ... ...
#C. felis         ... ... ... ... ... .A. A.. ... ... ... ... ... ..T T.. ... ...
#C. baileyi       ... ... ... ... ... .-. ... ... ... ... ... ..C ..- -.. ... ...
#C. muris         ... ... ... ... ... .AG ... G.. C.. ..G ... ..C ..- -.. ... ...
#C. andersoni     ... ... ... ... ... .AG ... G.. C.. ..G ... ..C ..- -.. ... ...
#C. serpentis     ... ... ... ... ... .AG ... G.. C.. ..G ... ..C ..- -G. ... ...
```

```
#C. hominis       TAA GAT AAG AAT AAT GAT TAA TAG GGA CAG TTG GGG GCA TTT GTA TTT
#C. parvum rabbit ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Isolate 2683     ... ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ...
#Isolate 2893     ... ... ..A .G. ... ... ... ... ... ... ... ... ... ... ... ...
#Dog 1640         ... ... .GA ... ... ... ... ... ... ... ... ... ... ... ... ...
#Dog 1653         ... ... .GA ... ... ... ... ... ... ... ... ... ... ... ... ...
#Dog 2884         ... ... .GA ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. canis         ... ... .GA ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. parvum pig    ... ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. meleagridis   ... ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. parvum bovine ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Horse 2469       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Horse 2468       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Horse 2467       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. wrairi        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. felis         ... ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. baileyi       ..G ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. muris         ..G ..C ..A .G. ... .G. ... ... ... ... ... ... ... ..C ... ...
#C. andersoni     ..G ..C ..A .G. ... .G. ... ... ... ... ... ... ... ..C ... ...
#C. serpentis     ..G ... ..A .G. ... .G. ... ... ... ... ... ... ... ..C ... ...
```

```
#C. hominis       AAC AGT CAG AGG TGA AAT TCT TAG ATT TGT TAA AGA CAA ACT AAT GCG
#C. parvum rabbit ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Isolate 2683     ... ... ... ... ... ... ... ... ... ... ... ... ... ... .G. ...
#Isolate 2893     ... ... ... ... ... ... ... ... ... ... ... ... ... ... .G. ...
#Dog 1640         ... ... T.. ... ... ... ... ... ... ... ... ... ... ... ... ...
#Dog 1653         ... ... T.. ... ... ... ... ... ... ... ... ... ... ... ... ...
#Dog 2884         ... ... T.. ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. canis         ... ... T.. ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. parvum pig    ... ... ... ... ... ... ... ... ... ... ... ... ... ... .G. ...
#C. meleagridis   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. parvum bovine ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Horse 2469       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Horse 2468       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Horse 2467       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. wrairi        ... ... ... ... ... ... ... ... ... ... ... ... ... ... .G. ...
#C. felis         ... ... ... ... ... T.. ... ... ... ... ... ... ... ... ... ...
#C. baileyi       ... ... ... ... ... ... ... ... ... ... ... ... ... ... .C. ...
#C. muris         ... ..C ... ... ... ... ... ... ... ... ... ... .G. ... .C. ...
#C. andersoni     ... ..C ... ... ... ... ... ... ... ... ... ... .G. ... .C. ...
#C. serpentis     ... ... ... ... ... ... ... ... ... ... ... ... .G. ... .C. ...
```

```
#C. hominis       AAA GCA TTT GCC AAG GAT GTT TTC ATT AAT CAA GAA CGA AAG TTA GGG
#C. parvum rabbit ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Isolate 2683     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Isolate 2893     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Dog 1640         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Dog 1653         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Dog 2884         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. canis         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. parvum pig    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. meleagridis   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C.parvum bovine ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Horse 2469       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Horse 2468       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Horse 2467       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. wrairi        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. felis         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. baileyi       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. muris         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. andersoni     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C. serpentis     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
```

```
#C. hominis       GAT CGA AGA CGA TC
#C. parvum rabbit ... ... ... ... ..
#Isolate 2683     ... ... ... ... ..
#Isolate 2893     ... ... ... ... ..
#Dog 1640         ... ... ... ... ..
#Dog 1653         ... ... ... ... ..
#Dog 2884         ... ... ... ... ..
#C. canis         ... ... ... ... ..
#C. parvum pig    ... ... ... ... ..
#C. meleagridis   ... ... ... ... ..
#C. parvum bovine ... ... ... ... ..
#Horse 2469       ... ... ... ... ..
#Horse 2468       ... ... ... ... ..
#Horse 2467       ... ... ... ... ..
#C. wrairi        ... ... ... ... ..
#C. felis         ... ... ... ... ..
#C. baileyi       ... ... ... ... ..
#C. muris         ... ... ... ... ..
#C. andersoni     ... ... ... ... ..
#C. serpentis     ... ... ... ... ..
```

Appendix B (ii)

Multiple sequence alignment of a small extrachromosomal, viral-like, dsRNA molecule from New Zealand *Cryptosporidium* isolates used in sub-genotyping.

```
#MEGA
!Title : dsRNA;
!Description  Extrachromosomal, small, viral-like dsRNA;
!Format
   DataType=Nucleotide CodeTable=Standard
   NSeqs=23 NSites=249
   Identical=. Missing=? Indel=-;


!Domain=Data property=Coding CodonStart=1;
#KSU-1       GGT TCA TGT AGT ACC GTG AAC AAG GCG CTT TAA ATA CCT AAT ATT TT-
#Wgtn_2217H ... ... ... ... ... A.. ..T ... ... ..A ... .AT A.C ..A ..A ..-
#Wgtn_2221H ... ... ... ... ... A.. ..T ... ... ..A ... .AT A.C ..A ..A ..-
#Wgtn_2222H ... ... ... ... ... A.. ..T ... ... ..A ... .AT A.C ..A ..A ..-
#Wgtn_2642H ... ... ... ... ... A.. ..T ... ... ..A ... .AT A.C ..A ..A ..-
#Wgtn_2651H ... ... ... ... ... A.. ..T ... ... ..A ... .AT A.C ..A ..A ..-
#Wgtn_2652H ... ... ... ... ... A.. ..T ... ... ..A ... .AT A.C ..A ..A ..-
#Wgtn_2641H ... ... ... ... ... A.. ..T ... ... ..A ... .AT A.C ..A ..A ..-
#Wgtn_2654H ... ... ... ... ... A.. ..T ... ... ..A ... .AT A.C ..A ..A ..-
#Wgtn_2655H ... ... ... ... ... A.. ..T ... ... ..A ... .AT A.C ..A ..A ..-
#Wgtn_2656H ... ... ... ... ... A.. ..T ... ... ..A ... .AT A.C ..A ..A ..-
#Wgtn_2653H ... ... ... ... ... A.. ..T ... ... ..A ... .AT A.C ..A ..A ..-
#Wgtn_2660H ... ... ... ... ... A.. ..T ... ... ..A ... .AT A.C ..A ..A ..-
#Wgtn_2721H ... ... ... ... ... A.. ..T ... ... ..A ... .AT A.C ..A ..A ..-
#Wgtn_2719H ... ... ... ... ... A.. ..T ... ... ..A ... .AT A.C ..A ..A ..-
#Wgtn_2657H ... ... ... ... ... A.. ..T ... ... ..A ... .AT A.C ..A ..A ..-
#Wgtn_2726H ... ... ... ... ... A.. ..T ... ... ..A ... .AT A.C ..A ..A ..-
#Wgtn_2214H ... ... ... ... ... A.. ..T ... ... ..A ... .AT A.C ..A ..A ..-
#BOP_2840B  ... ... ... ... ... A.. .G. ... ... ..- -.. G.. ..C ..G .AA ..-
#Sthd_2491B ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ..-
#BOP_2388B  ... ... ... ... ... ... ... ... ... ... ... G.. ... ... ... ..T
#Ham_2682B  ... ... ... ... ... ... ... ... ... ... ... G.. ... ... ... ..T
#Wgtn_2522B ... ... ... ... ... ... ... ... ... ... ... G.. ... ... ... ..T


#KSU-1       ATG ATT ACA AGT TTT GAA TCA ATA GAG AAA AAG AAT GCA GTT TAC TAT
#Wgtn_2217H ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2221H ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2222H ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2642H ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2651H ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2652H ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2641H ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2654H ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2655H ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2656H ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2653H ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2660H ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2721H ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2719H ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2657H ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2726H ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2214H ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#BOP_2840B  ... ... ... .A. ... ... C.. ..T ... ... ... ... ... ... ... ...
#Sthd_2491B ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#BOP_2388B  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Ham_2682B  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2522B ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
```

```
#KSU-1       CCA GTG GAT TTG AAA TTT GTC ACT GAC TTA TCT TCA GAT CTT TCG AAT
#Wgtn_2217H ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2221H ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2222H ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2642H ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2651H ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2652H ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2641H ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2654H ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2655H ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2656H ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2653H ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2660H ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2721H ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2719H ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2657H ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2726H ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2214H ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#BOP_2840B  ... ..A ... ... ... ... ... ... ... ... ... ..G ... ... ... ...
#Sthd_2491B ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#BOP_2388B  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Ham_2682B  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2522B ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

#KSU-1       ACA GCT GAC GGA TTA GGC CAG GCT TGG TAT AAA ATT TCA CAA GTC GCA
#Wgtn_2217H ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2221H ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2222H ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2642H ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2651H ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2652H ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2641H ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2654H ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2655H ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2656H ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2653H ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2660H ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2721H ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2719H ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2657H ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2726H ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2214H ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#BOP_2840B  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Sthd_2491B ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#BOP_2388B  ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Ham_2682B  ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Wgtn_2522B ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

#KSU-1       GTA GAG CAT ATA ATC CTA ACG GCA TTG AAG ATA AAT TAT GTC TTG AAT
#Wgtn_2217H ... ... ... ... ... T.. ..A ... ... ... ... ... ... ... ... ...
#Wgtn_2221H ... ... ... ... ... T.. ..A ... ... ... ... ... ... ... ... ...
#Wgtn_2222H ... ... ... ... ... T.. ..A ... ... ... ... ... ... ... ... ...
#Wgtn_2642H ... ... ... ... ... T.. ..A ... ... ... ... ... ... ... ... ...
#Wgtn_2651H ... ... ... ... ... T.. ..A ... ... ... ... ... ... ... ... ...
#Wgtn_2652H ... ... ... ... ... T.. ..A ... ... ... ... ... ... ... ... ...
#Wgtn_2641H ... ... ... ... ... T.. ..A ... ... ... ... ... ... ... ... ...
#Wgtn_2654H ... ... ... ... ... T.. ..A ... ... ... ... ... ... ... ... ...
#Wgtn_2655H ... ... ... ... ... T.. ..A ... ... ... ... ... ... ... ... ...
#Wgtn_2656H ... ... ... ... ... T.. ..A ... ... ... ... ... ... ... ... ...
#Wgtn_2653H ... ... ... ... ... T.. ..A ... ... ... ... ... ... ... ... ...
#Wgtn_2660H ... ... ... ... ... T.. ..A ... ... ... ... ... ... ... ... ...
#Wgtn_2721H ... ... ... ... ... T.. ..A ... ... ... ... ... ... ... ... ...
#Wgtn_2719H ... ... ... ... ... T.. ..A ... ... ... ... ... ... ... ... ...
#Wgtn_2657H ... ... ... ... ... T.. ..A ... ... ... ... ... ... ... ... ...
#Wgtn_2726H ... ... ... ... ... T.. ..A ... ... ... ... ... ... ... ... ...
#Wgtn_2214H ... ... ... ... ... T.. ..A ... ... ... ... ... ... ... ... ...
#BOP_2840B  A.. ... ... ... ... T.. ..A ... ... ... ... ... ... ... ... ...
#Sthd_2491B ... ... ... ... ... ..G ..A ... ... ... ... ... ... ... ... ...
#BOP_2388B  ... ... ... ... ... ..G ..A ... ... ... ... ... ... ... ... ...
#Ham_2682B  ... ... ... ... ... ..G ..A ... ... ... ... ... ... ... ... ...
#Wgtn_2522B ... ... ... ... ... ..G ..A ... ... ... ... ... ... ... ... ...
```

```
#KSU-1       CAT AGA ACC
#Wgtn_2217H ... ... ...
#Wgtn_2221H ... ... ...
#Wgtn_2222H ... ... ...
#Wgtn_2642H ... ... ...
#Wgtn_2651H ... ... ...
#Wgtn_2652H ... ... ...
#Wgtn_2641H ... ... ...
#Wgtn_2654H ... ... ...
#Wgtn_2655H ... ... ...
#Wgtn_2656H ... ... ...
#Wgtn_2653H ... ... ...
#Wgtn_2660H ... ... ...
#Wgtn_2721H ... ... ...
#Wgtn_2719H ... ... ...
#Wgtn_2657H ... ... ...
#Wgtn_2726H ... ... ...
#Wgtn_2214H ... ... ...
#BOP_2840B  ... ... ...
#Sthd_2491B ... ... ...
#BOP_2388B  ... ... ...
#Ham_2682B  ... ... ...
#Wgtn_2522B ... ... ...
```

Appendix B (iii)

Evolutionary genetic distances at the 18S rDNA locus, based on the number of nucleotide differences among *Cryptosporidium* species and *Cryptosporidium* genotypes.

```
Title: : 18S-msf.
Description
  No. of Taxa : 20
  Data Title : : 18S-msf.
  Data Type : Nucleotide (Coding)
  Analysis : Pairwise distance calculation
    Compute : Distances only
  Include Sites : ============================
    Gaps/Missing Data : Complete Deletion
    Codon Positions : 1st+2nd+3rd+Noncoding
  Substitution Model : ============================
    Model : Nucleotide: Number of differences
    Substitutions to Include : d: Transitions + Transversions
    Pattern among Lineages : Same (Homogeneous)
    Rates among sites : Uniform rates
  No. of Sites : 687
  d : Estimate

[ 1] #C. hominis
[ 2] #C. parvum rabbit
[ 3] #C. meleagridis
[ 4] #C. parvum bovine
[ 5] #Horse isolate 2469
[ 6] #Horse isolate 2468
[ 7] #Horse isolate 2467
[ 8] #C. parvum pig
[ 9] #C. wrairi
[10] #Human isolate 2683
[11] #Human isolate 2893
[12] #Dog isolate 1640
[13] #Dog isolate 1653
[14] #Dog isolate 2884
[15] #C. canis
[16] #C. felis
[17] #C. baileyi
[18] #C. serpentis
[19] #C. andersoni
[20] #C. muris

[      1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19 20 ]
[ 1]
[ 2]   2
[ 3]   3  3
[ 4]   3  3  4
[ 5]   3  3  4  0
[ 6]   3  3  4  0  0
[ 7]   3  3  4  0  0  0
[ 8]   6  8  5  7  7  7  7
[ 9]   7  7  6  4  4  4  4  7
[10]  12 14 11 13 13 13 13  6 13
[11]  13 15 12 14 14 14 14  7 14  1
[12]  16 16 13 15 15 15 15 14 17 12 13
[13]  16 16 13 15 15 15 15 14 17 12 13  0
[14]  15 15 12 14 14 14 14 13 16 11 12  1  1
[15]  15 15 12 14 14 14 14 13 16 11 12  1  1  0
[16]  16 18 15 17 17 17 17 14 17 15 16 19 19 18 18
[17]  28 28 26 27 27 27 27 28 29 29 30 28 28 27 27 27
[18]  44 44 41 43 43 43 43 43 43 45 44 48 48 47 47 46 36
[19]  50 50 47 49 49 49 49 49 49 50 49 54 54 53 53 52 42  8
[20]  51 51 48 50 50 50 50 50 50 51 50 53 53 52 52 53 41 12  4
```

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