Sequence and PCR–RFLP analysis of the internal transcribed spacers of the rDNA repeat unit in isolates of *Cryptosporidium* from different hosts

U. M. MORGAN[1]*, P. DEPLAZES[2], D. A. FORBES[3], F. SPANO[4], H. HERTZBERG[2], K. D. SARGENT[1], A. ELLIOT[1] *and* R. C. A. THOMPSON[1]

[1] *World Health Organization Collaborating Centre for the Molecular Epidemiology of Parasitic Infections and State Agricultural Biotechnology Centre, Division of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, WA 6150, Australia*
[2] *Institute of Parasitology, University of Zurich, Winterthurerstrasse 266a, CH-8057, Zurich, Switzerland*
[3] *Department of Paediatrics, University of Western Australia, Princess Margaret Hospital, GPO Box D184, WA 6001, Australia*
[4] *Istituto di Parassitologia, Università di Roma 'La Sapienza', P. le A. Moro 5, 00185 Rome, Italy*

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SUMMARY

The *Cryptosporidium* ITS1, 5·8S and ITS2 rDNA regions from a number of *Cryptosporidium* isolates from different hosts and geographical areas were cloned and sequenced in order to investigate the extent of sequence heterogeneity between human and cattle-derived isolates from different geographical locations and also between isolates of *Cryptosporidium* from different hosts such as cats, pigs, mice and a koala. Calf-derived isolates from different continents were virtually identical as were human-derived isolates from the UK and Australia. Genetic differences between *Cryptosporidium* isolates were extensive and were in fact greater than the level of nucleotide divergence between *Toxoplasma gondii* and *Neospora caninum* rDNA sequences. Based on the sequence information derived from this study, PCR–RFLP of the ITS1 region was undertaken in order to directly amplify and genotype *Cryptosporidium* isolates from different hosts. This PCR–RFLP approach can now be used for molecular epidemiology studies, circumventing the need for costly sequencing and allowing a wider range of genetically different isolates to be examined.

Key words: *Cryptosporidium*, rDNA, ITS, genotypes.

INTRODUCTION

The emergence of *Cryptosporidium* as an important cause of diarrhoeal illness, and its increasing role in both localized and widespread outbreaks of disease, is a public health problem of global proportions for both developed and developing countries (Fayer, Speer & Dubey, 1997). The application of PCR-based techniques will greatly enhance diagnosis of this ubiquitous parasite. However, the diagnostic primers which are currently available are limited in the range of genotypes they can detect, and are only useful on a routine basis for discriminating between the genotypes identified in livestock and humans ('cattle' and 'human') (Peng *et al*. 1997; Spano *et al*. 1997, 1998; Morgan & Thompson, 1998). Other genotypes from animals such as cats, pigs, rodents and marsupials are genetically very different from the 'cattle' and 'human' genotypes (Morgan *et al*. 1998*a*). This has posed problems in developing 'universal' diagnostic primers with the capability of identifying all genotypes of *C. parvum*. It is very important, particularly in outbreak situations, to be able to determine the sources of infection and environmental contamination. The possible genotype(s) involved must be identified and/or excluded, especially those of domestic or wild animal origin. Although this can be achieved using sequence analysis (Morgan *et al*. 1998*a*), this is costly and impractical for routine diagnosis and epidemiological investigations. There is therefore a need to develop a PCR-based 'typing' approach which can be applied directly to clinical or environmental samples in order to identify the particular genotype(s) of *Cryptosporidium* present.

Previous research in our laboratory has identified differences between isolates of *Cryptosporidium* of human and cattle origin as well as between isolates from pigs, cats, mice and marsupials using sequence analysis of the conserved 18S rDNA gene (Morgan *et al*. 1997*a*, 1998*a*). The aims of the present study were to investigate the extent of sequence heterogeneity between isolates of *Cryptosporidium* from a variety of hosts for the less conserved ITS1 and ITS2 ribosomal DNA (rDNA) regions and to determine if these differences were conserved in isolates of *Cryptosporidium* from different geographical regions. The rDNA repeat unit of *Crypto-*

* Corresponding author: Division of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, WA 6150. Tel: +08 9360 2457. Fax: +08 9310 4144. E-mail: morgan@numbat.murdoch.edu.au

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sporidium has recently been characterized (Le Blancq *et al*. 1997). Two distinct types were identified, designated Type A and Type B (Le Blancq *et al*. 1997). A second aim of the study therefore was to attempt to characterize a portion of the Type B ribosomal unit from human and animal-derived isolates of *Cryptosporidium*. Finally, we wished to develop a molecular epidemiological tool for genotype identification involving RFLP analysis of the ITS1 region.

MATERIALS AND METHODS

Sources of parasite isolates, DNA purification and primer design

Sources of parasite isolates are listed in Table 1. Isolates were derived directly from their host of origin with the exception of isolates A1, S1 and L1 which were passaged in sheep prior to this study. DNA was purified as previously described (Morgan *et al*. 1997*a*). Primers were designed using Amplify 2.1 (Bill Engels, University of Wisconsin), and oligonucleotides were synthesized by Gibco BRL (Gaithersburg, MD, USA).

PCR amplification, cloning and sequencing of the ITS1, 5·8S and ITS 2 Type A rDNA region from calf, human, cat, pig, mouse and koala-derived isolates

A forward primer was designed based on available rDNA 18S sequence information for *Cryptosporidium* and a reverse primer developed by aligning available apicomplexan 28S rDNA sequences (Van de Peer *et al*. 1994). The primers, designated 18SF+ (5′ TGAATATGCATCGTGATGG 3′) and 28SR1 (5′ CTGATATGCTTAAGTTC 3′), amplified a fragment encompassing the ITS1, 5·8S and ITS2 rDNA regions. PCR reaction mixtures consisted of 12·5 pmoles of each primer, 200 μM of each dNTP, 2 mM $MgCl_2$, 67 mM Tris–HCl (pH 8·8), 16·6 mM $(NH_4)_2SO_4$, and 0·1 unit *Tth* Plus DNA polymerase (Fisher Biotech, Western Australia). Reactions were performed on a PE 2,400 (Perkin Elmer, Foster City, California) thermal cycler. The amplification programme consisted of 1 preliminary cycle of 96 °C for 2 min, 58 °C for 1 min and 60 °C for 3 min, followed by 45 cycles of 94 °C for 30 sec, 58 °C for 30 sec and 60 °C for 2 min with a final cycle of 60 °C for 7 min. PCR products were cloned into the PCR 2000™ T-vector (Invitrogen, USA) and transformants screened by PCR. Positive colonies were sequenced using an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions except that the annealing temperature was raised to 60 °C. At least 3 clones of each PCR product were sequenced in both directions. Sequences were analysed using SeqEd v 1.0.3. (Applied Biosystems) and aligned using the Clustal V (Higgins, Bleasby & Fuchs, 1991), sequence alignment programme. Based on the sequence information generated from these clones, an additional primer, ITSR1 (5′ GAATTATGCAGTTCACATTGC 3′), was designed at the start of the 5·8S gene to amplify the ITS1 region in combination with the 18S+ forward primer. Expected restriction fragment sizes for the ITS1 region using the endonuclease *Dra* I were determined using DNA Strider 1.0.

Sequence and PCR analysis of the Type B rDNA unit

The oligonucleotides Cp3 (5′ CGTCTATCAGTGGGTTTTGAA 3′) and Cp4 (5′ CTTTCCCTCACGGTACTTGTT 3′) were shown to specifically amplify a 514 bp product from the Type B rDNA unit from the 'cattle' *C. parvum* genotype (Le Blancq *et al*. 1997). We employed these primers in an attempt to amplify the Type B ribosomal unit from a number of *Cryptosporidium* isolates of distinct host origins. The cycling conditions were 96 °C for 2 min, 58 °C for 1 min and 68 °C for 2 min, followed by 50 cycles of 94 °C for 30 sec, 58 °C for 20 sec and 68 °C for 45 sec with a final cycle of 68 °C for 7 min. The amplicons obtained from a human (H1) and a calf (C13) isolate were cloned and sequenced as described above and aligned to the KSU-1 sequence (Le Blancq *et al*. 1997), using the Clustal V (Higgins *et al*. 1991) sequence alignment programme.

Phylogenetic analysis of sequence information

A phylogenetic analysis based on the nucleotide sequences of the ITS1, 5·8S and ITS2 regions from different isolates was conducted using PHYLIP 3.5p (Felsenstein, 1989). A similarity index among *Cryptosporidium* isolates was created using the formula for Kimura's Distance. Phenograms were constructed from genetic distance matrices using the Unweighted Pair-Group Method (UPGMA) and DRAWGRAM programs available in PHYLIP 3.5p (Felstenstein, 1989). The rDNA sequences of the KSU-1 isolates of *C. parvum* previously reported by Le Blancq *et al*. (1997) (Genebank accession numbers: AF015772, AF015773, AF015774) and Zhu, Marchewska & Keithly (1998 unpublished) (Genebank accession number AF040725) were included in the analysis together with the rDNA sequences from *Toxoplasma gondii* and *Neospora caninum* (Payne & Ellis, 1996, Genebank accession numbers L49390 and L49389 respectively).

PCR–RFLP analysis of the ITS1 rDNA region

PCR reactions for amplification of the ITS1 rDNA region using the primers 18SF+ and ITSR1

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Table 1. Isolates of *Cryptosporidium* used in this study

(AgWA, Agriculture Western Australia; CSIRO, Commonwealth Scientific and Industrial Research Organisation, Victoria; CVL, Central Veterinary Laboratories, Adelaide; IDR, Institute of Parasitology, Rome, Italy; IFP, Institut für Parasitologie, Zurich, Switzerland; MA, Ministry of Agriculture, Department of Veterinary Services, Nicosia, Cyprus; MAH, Moredun Animal Health Ltd; MU, Murdoch University, Western Australia; PMH, Princess Margaret Hospital, Perth; UND, University of North Dakota School of Medicine, North Dakota, USA; VP Veterinary Pathology, Perth.)

Code	Host	Genotype*	Geographical origin	CP3/Cp4 +/−	Source
H1	Human	Human[1,2]	Perth, WA	+	PMH
H4	Human	Human[2]	Perth, WA	−	PMH
H16	Human	Human[1]	Perth, WA	+	PMH
H18	Human	Calf[1,2]	Perth, WA	+	PMH
H19	Human	Human[3]	Perth, WA	+	PMH
H20	Human	Human[3]	Perth, WA	+	PMH
H21	Human	Human[3]	Perth, WA	+	PMH
H24	Human	Human[3]	Perth, WA	−	SHL
P12	Human	Human[4]	Wales	−	IDR
P18	Human	Human[4]	Central England	−	IDR
P29	Human	Human[4]	South England	−	IDR
A1	Alpaca	Calf[4]	Peru	+	IDR
S1	Sheep	Calf[4]	Spain	+	IDR
C1	Calf	Calf[1,2]	Millicent, SA	+	CVL
C2	Calf	Calf[1,2]	Lucindale, SA	+	CVL
C6	Calf	Calf[1]	Willunga, SA	+	CVL
C7	Calf	Calf[1]	Penola SA	+	CVL
C13	Calf	Calf[1]	USA	+	UND
sC26	Calf	Calf[5]	Switzerland	−	IFP
sC33	Calf	Calf[5]	Switzerland	−	IFP
G1	Goat	Calf[5]	Toodyay, WA	+	AgWA
G3	Goat	Calf[5]	Cyprus	−	MA
L1	Deer	Calf[1,2]	Edinburgh, Scotland	+	MAH
M24	Mouse	Mouse[5]	Walpeup, Victoria	−	CSIRO
M26	Mouse	Mouse[5]	Victoria	−	CSIRO
K1	Koala	Koala[5]	SA	−	CVL
Pig1	Pig	Pig[5]	Switzerland	−	EFP
Pig2	Pig	Pig[5]	Popanyinning, WA	−	MU
Pig3	Pig	Pig[5]	Popanyinning, WA	−	MU
Pig4	Pig	Pig[5]	Albany, WA	−	AgWA
Pig5	Pig	N.D.P.	Albany WA	−	AgWA
Ct1	Cat	*C. felis*[6]	Perth, WA	−	MU
Ct2	Cat	*C. felis*[6]	Perth, WA	−	MU
Ct3	Cat	*C. felis*[7]	Spearwood, WA	−	MU
Ct4	Cat	*C. felis*[7]	Morley, WA	−	VP
Ct5	Cat	*C. felis*[7]	Subiaco, WA	−	VP
Ct6	Cat	*C. felis*[7]	Perth, WA	−	MU

* [1] Morgan *et al.* 1997; [2] Morgan *et al.* 1995; [3] RAPD analysis (unpublished); [4] Spano *et al.* 1997; [5] Morgan *et al.* 1988*a*; [6] Sargent *et al.* 1998; [7] Morgan *et al.* 1998*c*. N.D.P., Not determined prior to this study.

consisted of 6·25 pmoles of each primer, 200 μM of each dNTP, 1·25 mM $MgCl_2$, 67 mM Tris–HCl (pH 8·8), 16·6 mM $(NH_4)_2SO_4$ and 0·1 unit *Tth* Plus DNA polymerase (Fisher Biotech). Reactions were performed on a PE 2,400 (Perkin Elmer, Foster City, California) thermal cycler. The reaction programme consisted of 1 preliminary cycle of 96 °C for 2 min and 61 °C for 3 min, followed by 50 cycles of 94 °C for 30 sec and 61 °C for 45 sec with a final cycle of 61 °C for 7 min. PCR products (7·5 μl) were digested unpurified in a reaction containing 15 units of *Dra* I (Promega, USA), 1 × reaction buffer, 4 μg BSA and sterile distilled water to a final volume of 40 μl. Digestions were incubated at 37 °C from 3 h to overnight, electrophoresed on 3·5% MetaPhor agarose (FMC®, Rockland, USA) and post-stained with ethidium bromide.

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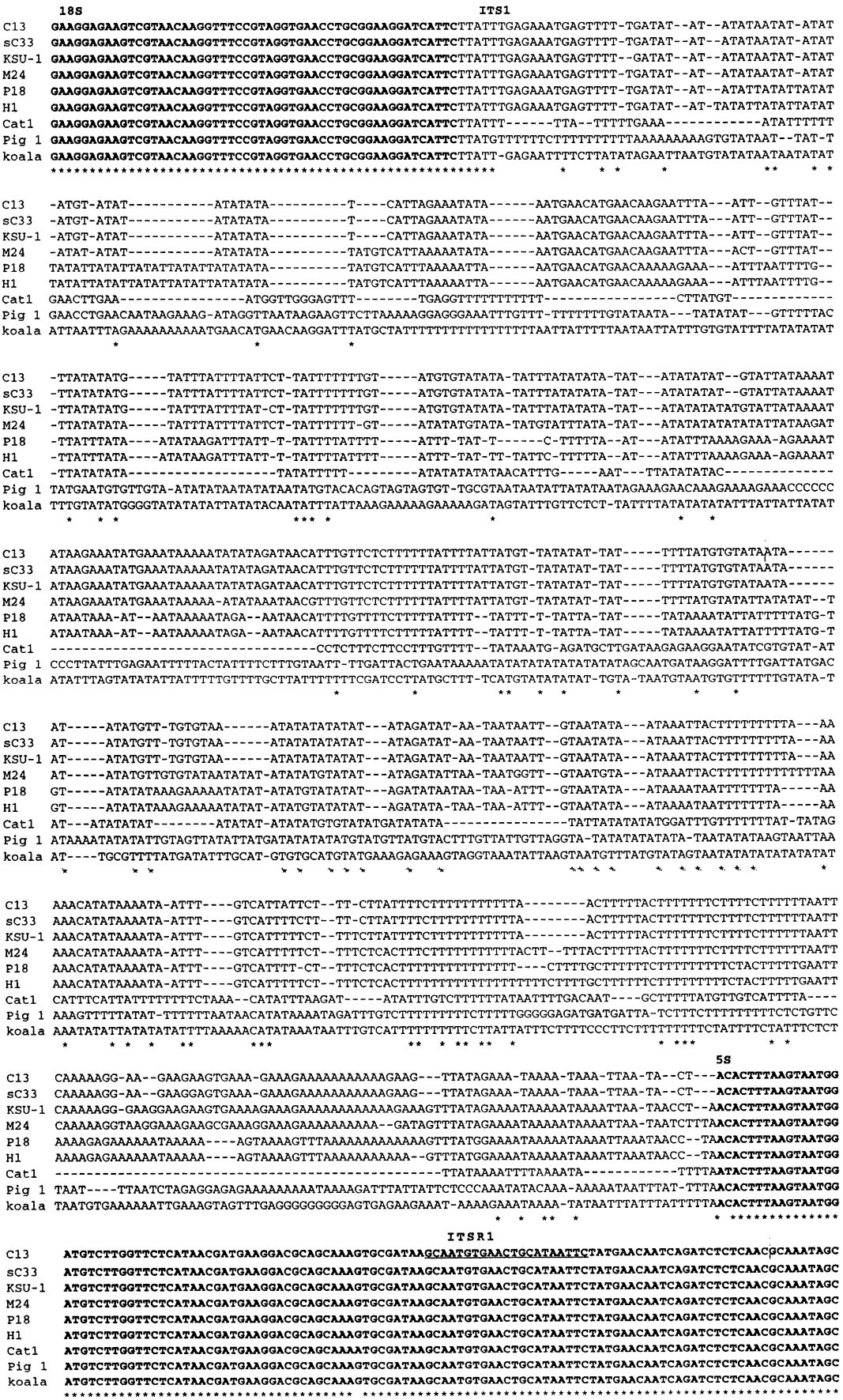

Fig. 1. For legend see opposite page.

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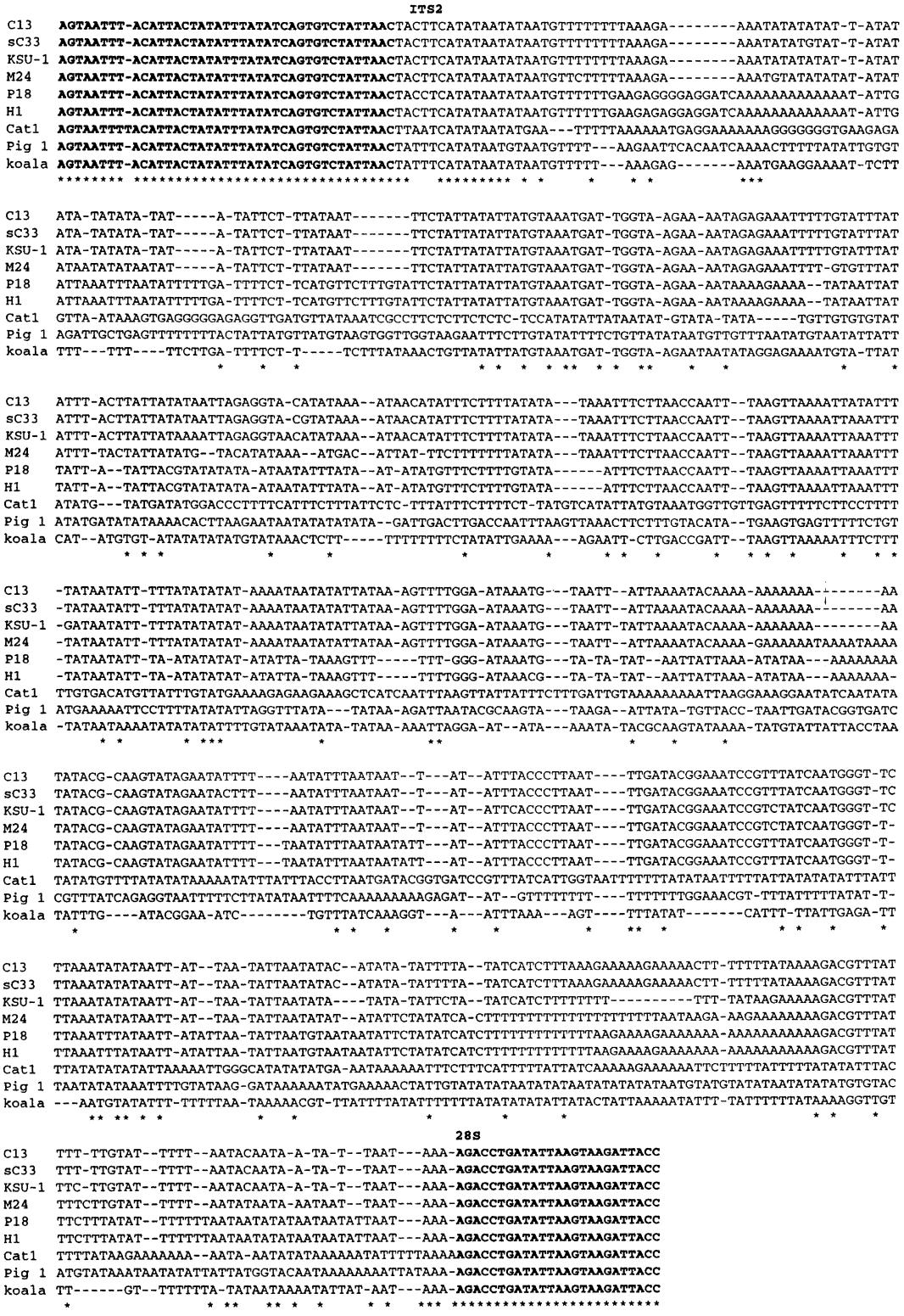

Fig. 1. Sequence alignments of the *Cryptosporidium* rDNA ITS1, 5·8S, and ITS2 regions from various isolates. (KSU-1 isolate was previously sequenced by Le Blancq *et al*. 1997.) Primers 18S+ and 285 R1 not included. GenBank Accession numbers for these sequences are as follows: [C13-AF093008], [sC33-AF093009], [M24-AF093010], [P18-AF093011], [H1-AF093012], [Cat 1-AF093013], [Pig 1-AF093014], [Koala-AF093015].

RESULTS

Sequence analysis of the ITS1, 5·8S and ITS2 Type A regions from various hosts

A genomic DNA region of approximately 1600 bp encompassing the ITS1, 5·8S and ITS2 sequences of the Type A ribosomal unit was amplified with primers 18SF+ and 28SR1 from 8 *Cryptosporidium* isolates of various host origin: C13 and sC33 (cattle), P18 and H1 (human), M24 (mouse), Ct1 (cat), Pig1 (pig) and K1 (koala). Sequence comparison revealed a very substantial degree of variation in the ITS1

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Table 2. Length and percentage AT richness of the ITS1 and ITS2 rDNA regions of isolates of *Cryptosporidium* from different hosts

Isolate	Host	Length of ITS1 (bp)	ITS1– % AT rich	Length of ITS2 (bp)	ITS2– % AT rich
C13	Cattle	496	88	508	88
Ct1	Cat	363	83	585	80
P18	Human	523	91	566	88
K1	Koala	618	84	497	86
M24	Mouse	534	88	518	88
Pig1	Pig	607	82	479	79

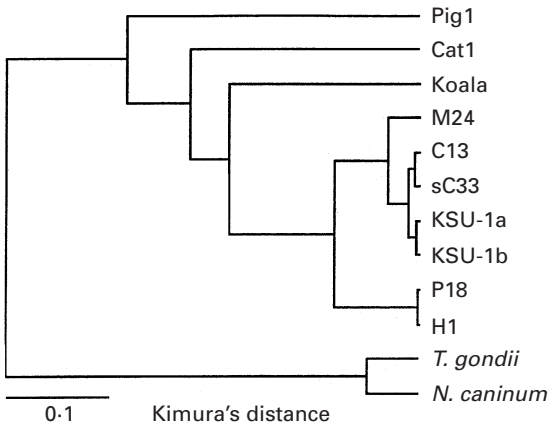

Fig. 2. Phylogram of Kimura's distance generated from the rDNA ITS1, 5·8S, and ITS2 regions from human, cattle, koala, pig, cat and mouse-derived isolates of *Cryptosporidium*. (KSU-1a–Le Blancq *et al*. 1997; KSU-1b–Zhu *et al*. 1998, unpublished.) Additional sequence from *Toxoplasma gondii* and *Neospora caninum* (Payne & Ellis, 1996, Genebank accession numbers L49390 and L49389 respectively), were included as a comparison. (Le Blancq *et al*. (1997) Gene bank accession numbers: AF015772, AF015773, AF015774; Zhu *et al*. (1998) unpublished, Genebank accession number AF040725.)

and ITS2 regions between the different isolates (Fig. 1). However, there was minimal variation between isolates from the same host species (Fig. 1). The extent of intra-individual variation, ascertained by sequencing numerous PCR-derived clones from the same isolate was observed to be low and was consistent with the minor differences seen between cattle isolates (C13, sC33 and KSU-1) (Fig. 1). There were substantial length differences particularly in the ITS1 region between the different isolates (Table 2), with the cat isolate (Ct1) exhibiting the shortest ITS1 region (363 bp) and the koala isolate (K1) the longest (618 bp). The ITS2 region ranged in size from 479 bp (Pig1) to 585 bp (Ct1). The percentage AT richness for the ITS1 and ITS2 ranged from 79 to 91 % (Table 2). The cattle and the human isolates showed an overall sequence divergence of approximately 28 and 25 % within the ITS1 and ITS2 regions respectively. Notably, these values are greater than the extent of sequence divergence between *T. gondii* and *N. caninum* for the ITS1 (24 %). The 5·8S region is 155 bp in length in all *Cryptosporidium* isolates and is approximately 75 % AT rich. Percentage differences for the other isolates were difficult to calculate accurately due to the amount of sequence gaps between isolates but were much larger than the percentage differences between human and cattle isolates.

Phylogenetic analysis of ITS1, 5·8S and ITS2 rDNA regions

Phylogenetic analysis produced 6 major groups (Fig. 2). A 'human' group consisting of the 2 human (H1, P18) isolates, a 'cattle' group which contained all the cattle isolates (C13; sC33 and KSU) and a 'pig', 'mouse', 'cat' and 'koala' group. The mouse isolate (M24) was most closely related to the calf isolates whereas all the others were genetically very distinct (Fig. 2). Differences were observed between the 2 sequences reported for the KSU-1 isolate by Le Blancq *et al*. (1997), (KSU-1a) and by Zhu *et al*. (1988), (KSU-1b). This probably reflects intra-individual variation (estimated at 2 % by Le Blancq *et al*. 1997), and possibly minor differences due to inter-laboratory sequencing accuracy. Carraway, Tzipori & Widmer (1996) have also sequenced the rDNA ITS1 region from a number of different *C. parvum* isolates, including a UK deer isolate (L1) which has been characterized as a 'cattle' genotype (Carraway, Tzipori & Widmer, 1997; Morgan *et al*. 1995, 1997*a*) and a US human isolate GCH2 characterized as a 'human' genotype (Carraway *et al*. 1997). Comparison of ITS1 sequence information for these isolates (L1; GCH2) with human (H1) and cattle (C1) isolates and subsequent phylogenetic analysis grouped the 2 human isolates together and the calf and deer isolates together (data not shown). Again minor sequence differences were observed, however, compared to the overall levels of variation, these differences were minimal and add strength to the concept that the 'cattle' and 'human' genotype is conserved across different geographical regions. Phylogenetic analysis using both Neighbour Joining and Parsimony methods revealed similar genetic pictures (data not shown).

Sequence and PCR analysis of the Type B rDNA unit

Le Blancq *et al*. (1997), reported the existence in *C. parvum* of 2 classes of distinct rDNA units, designated Type A and B. The same authors showed that the primers Cp3 and Cp4, encompass a region that spans 143 bp region at the 3′ end of the ITS2 and the first 371 bp at the start of the 28S gene, and that the primers specifically amplified a 514 bp product from the Type B unit of KSU-1 calf isolate. Using

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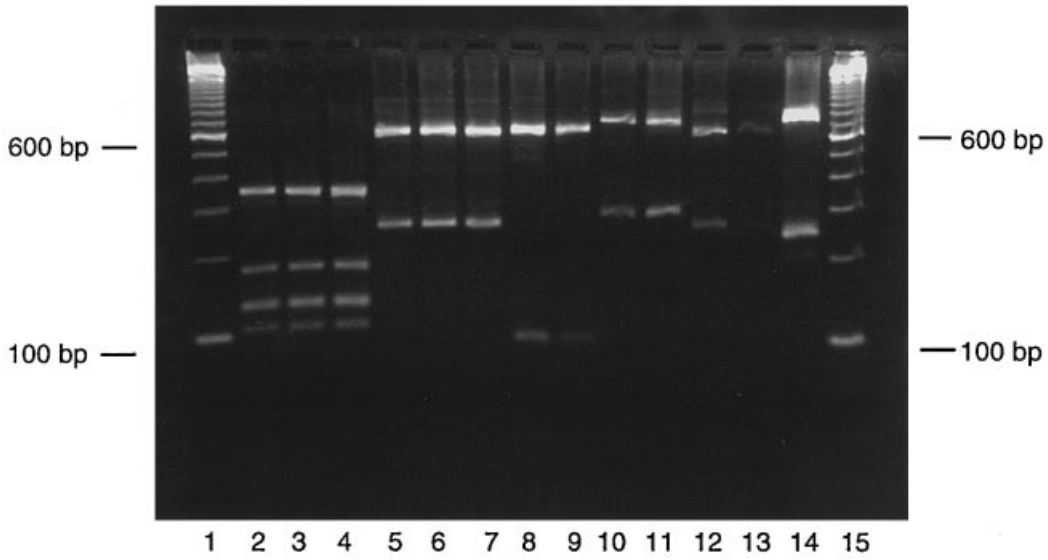

Fig. 3. PCR–RFLP analysis of the rDNA ITS1 region for a variety of isolates of *Cryptosporidium*. Lane 1, molecular weight marker*; lane 2, H19; lane 3, H20; lane 4, H21; lane 5, C1; lane 6, C2; lane 7, L1; lane 8, Ct1; lane 9, Ct2; lane 10, Pig1; lane 11, Pig2; lane 12, M26; lane 13, M24; lane 14, K1; lane 15, molecular weight marker. *100 bp ladder (Gibco BRL).

Table 3. 18S+/ITSR1 amplicon lengths and *Dra* I restriction fragment sizes for the ITS1 region of various isolates of *Cryptosporidium*

Isolate	Host	Size of PCR product (bp)	No. of *Dra* I sites (TTT/AAA)	Expected restriction fragment sizes (bp)
C13	Cattle	847	1	598, 249
Ct1	Cat	714	1	617, 97
H1	Human	884	4	348, 174, 132, 126, 104
K1	Koala	970	1	721, 249
M24	Mouse	884	1	625, 259
Pig1	Pig	958	1	670, 288

primers Cp3/Cp4, we amplified a PCR product of the expected molecular size from a calf (C13) and a human (H1) isolate. The C13 isolate shared 100% sequence identity with the KSU-1 Type B sequence. The H1 isolate, however, was virtually identical to the Type A sequence obtained for this isolate (data not shown). PCR screening of a range of isolates using the Cp3/Cp4 primer combination revealed that the 514 bp product could not be amplified from all isolates of *Cryptosporidium* even when the template concentration and the number of cycles were increased. The Cp3/Cp4 PCR product could only be amplified from some human (6/11) and cattle (9/12) isolates and from none of the cat, pig, koala or mouse-derived isolates (see Table 1).

PCR–RFLP analysis of the ITS1 rDNA region

RFLP analysis of the ITS1 region was conducted on a total of 37 isolates of *Cryptosporidium*, (see Table 1). PCR products amplified with primers 18SF+ and ITSR1 were digested with the endonuclease *Dra* I and the digestion products resolved by agarose gel electrophoresis. A representative gel showing RFLP profiles of the major genetic groups is shown in Fig. 3. RFLP analysis yielded 6 specific and reproducible fingerprint profiles for the different genetic groups; ('human', 'cattle', 'pig', 'mouse', 'koala' and 'cat'). The electrophoretic profiles observed were consistent with the sizes of the *Dra* I restriction fragments predicted using DNA Strider (see Table 3), with the exception of the human isolates. For human isolates, 5 restriction fragments were predicted, but in all digests only 4 bands could be seen, presumably due to the 132 bp and 126 bp bands co-migrating. Polyacrylamide gel electrophoresis could resolve these bands, however, as the human isolates were clearly distinct from all other isolates, this is not necessary.

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DISCUSSION

Molecular phylogenies of closely related species are usually obtained with rapidly evolving DNA regions (Schlotterer *et al*. 1994). The rDNA ITS regions have been shown to be suitable universal genomic sequences as they are present in all eukaryotes, can easily be amplified with primers in the highly conserved flanking regions and they show a high rate of divergence compared to coding regions of rDNA. Unlike *T. gondii*, in which each haploid genome contains approximately 110 copies of the rDNA unit (Guay *et al*. 1993), *Cryptosporidium* has been shown to possess 4 Type A and 1 Type B rDNA units per haploid genome (Le Blancq *et al*. 1997). However, despite their relatively low copy number, the rDNA units of *Cryptosporidium* are still a suitable target for the detection and typing of environmental samples where the numbers of oocysts recovered are usually very low since the ITS1 PCR product was readily amplified from all isolates examined.

Our previous studies have identified differences between human and animal isolates of *Cryptosporidium* using sequence analysis of the conserved 18S rDNA gene, the acetylCoA synthase gene and PCR analysis of an unidentified genomic fragment (Morgan *et al*. 1997*a*, 1998*a*, *b*, *c*; Sargent *et al*. 1998). In this study, the extent of sequence variation within the ITS1, 5·8S, and ITS2 regions between cattle (C13, sC33) and human isolates (P18, H1) from different geographical locations and from a pig (Pig1), cat (Ct1), koala (K1) and mouse isolate (M24), was determined by extensive sequence analysis of the Type A ribosomal unit. The present results have shown that the sequence divergence between human and various animal isolates in this region is considerable, yet cattle isolates from different geographical locations (C13, sC33) were virtually identical. A previously sequenced calf isolate (KSU-1) (Le Blancq *et al*. 1997; Zhu *et al*. 1998) was also included in the analysis and grouped very closely with the other calf isolates. ITS1 sequences for a deer isolate from the UK and a human isolate from the US, previously sequenced (Carraway *et al*. 1996), grouped with cattle and human genotypes respectively. Similarly, human isolates from Australia and the UK were also virtually identical for this region. This supports the concept that the 'cattle' and 'human' genotype is conserved across different geographical regions. The degree of sequence conservation among *Cryptosporidium* isolates of either 'human' or 'cattle' genotype from disparate geographical regions further supports the concept of a clonal population structure for this parasite (Morgan *et al*. 1997*b*).

Analysis of the ITS1, 5·8S and ITS2 Type A regions from a pig (Pig1), cat (Ct1), koala (K1) and mouse isolate (M24) has revealed even greater genetic divergence between these isolates than between the human and cattle isolates, with the exception of M24, as phylogenetic analysis revealed this isolate to be most closely related to the 'cattle' genotype. However, because the variation between the different genetic groups was so large, it is difficult to infer accurate phylogenies. The phylogram which depicts the differences in the ITS1, 5·8S and ITS2 regions between these isolates, is intended merely to show the extensive heterogeneity between isolates of *Cryptosporidium* from different hosts. Future phylogenetic analysis should concentrate on the more conserved 18S gene and also non-rDNA targets as these are likely to yield more phylogenetically meaningful data. The excessive divergence, for phylogenetic purposes observed among the ITS regions of several groups of *Cryptosporidium* isolates, coupled with the fact that the amount of variation between the different genetic groups was greater than that seen between *T. gondii* and *N. caninum* lends further support to the concept of numerous distinct species within *C. parvum*. However, it also raises questions as to the validity of *Neospora* as a genus distinct from *Toxoplasma*.

The use of primers Cp3/Cp4 (Le Blancq *et al*. 1997), designed on the Type B ITS2 and 28S regions, respectively, allowed us to amplify a specific Type B PCR product from a calf isolate (C13) which was 100% identical to the KSU-1 Type B unit. The H1 isolate, however, was virtually identical to the Type A sequence obtained for this isolate, indicating that the Cp3/Cp4 primers are diagnostic only for Type B units from 'cattle' genotypes. Notably, the Cp3/Cp4 primers yielded no PCR amplification from some of the human and cattle isolates, and from none of the other animal-derived isolates. These results suggest that extensive differences in the Type B rDNA may exist among *Cryptosporidium* isolates. The possibility of some *Cryptosporidium* isolates lacking the Type B rDNA unit as a result of large DNA rearrangements should also be considered. The sequence heterogeneity, or even the lack, of the Type B rDNA unit, inferred from our PCR analysis deserves further investigation, as it may provide the molecular basis for future genotyping methods able to discriminate among isolates belonging to the same genetic group.

RFLP analysis of the ITS1 region, using the enzyme *Dra* I, identified 6 distinct genotypes: 'human' 'cattle', (which comprises isolates from cattle, sheep, goat and an alpaca) 'pig', 'cat', 'koala' and 'mouse'. This molecular approach can now be used as a rapid and inexpensive method of genotyping *Cryptosporidium* isolates. RFLP analysis grouped the 9 human isolates into the 'human' genetic group, with the exception of the human isolate H18 which had previously been shown to display the 'calf' genotype (Morgan *et al*. 1997*a*, 1998*a*, *b*). A total of 12 isolates, 7 from cattle, 2 from goats, 1 sheep isolate, 1 alpaca isolate and 1 deer

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isolate were all grouped into the 'cattle' genotype by RFLP analysis. The 5 pig isolates analysed all displayed identical electrophoretic profiles. One of the pig isolates was from Switzerland, 2 were from Popanyinning near Perth, and the remaining 2 pig isolates were from an outbreak in Albany, some 402 km from Perth, confirming the widespread distribution of this genotype. All 6 cat isolates included in this study produced identical profiles as did the 2 mice isolates analysed. The koala isolate was totally distinct from all others. Occasionally, faint background bands were observed in the restriction profile of some isolates of a definite genetic group. However, these additional bands were not observed when RFLP analysis was performed on individually cloned PCR fragments, suggesting that the faint bands probably represented non-specific amplification products. The high levels of variation within the ITS1 region demonstrates the utility of this genetic locus in epidemiological studies, although more informative loci may reveal further distinguishable types.

The epidemiological significance and importance of these genotypes in relation to human infection has yet to be fully elucidated. However, the present research and that of others (Awad-El-Kariem *et al*. 1995; Carraway *et al*. 1997; Morgan *et al*. 1995, 1997*a*; 1998*a*, *b*; Peng *et al*. 1997; Spano *et al*. 1997, 1998; Vasquez *et al*. 1996) provides strong evidence that humans are susceptible to infection with isolates from livestock. The susceptibility of humans to infection with isolates from cats, pigs, mice and marsupials may be doubtful and requires further comparative studies with isolates from diverse geographical locations and also with isolates from immunocompromised and immunocompetent human hosts. In addition, the data presented here and in other studies clearly indicate that *C. parvum* is not a uniform species and that a taxonomic revision of the genus is necessary.

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REFERENCES

AWAD-EL-KARIEM, F. M., ROBINSON, H. A., DYSON, D. A., EVANS, D., WRIGHT, S., FOX, M. T. & McDONALD, V. M. (1995). Differentiation between human and animal strains of *Cryptosporidium parvum* using isoenzyme typing. *Parasitology* **110**, 129–132.

CARRAWAY, M., TZIPORI, S. & WIDMER, G. (1996). Identification of genetic heterogeneity in the *Cryptosporidium parvum* ribosomal repeat. *Applied and Environmental Microbiology* **62**, 712–716.

CARRAWAY, M., TZIPORI, S. & WIDMER, G. (1997). New RFLP marker in *Cryptosporidium parvum* identifies mixed parasite populations and genotypic instability in response to host change. *Infection and Immunity* **65**, 3958–3960.

FAYER, R., SPEER, C. A. & DUBEY, J. P. (1997). The general biology of *Cryptosporidium*. In *Cryptosporidium and Cryptosporidiosis* (ed. Fayer, R.), pp. 1–42. CRC Press, Boca Raton.

FELSENSTEIN, J. (1989). PHYLIP. Phylogeny interface package (version 3.2). *Cladistics* **5**, 164–166.

GUAY, J. M., DUBOIS, D., MORENCY, M. J., GAGNON, S., MERCIER, J. & LEVESQUE, R. C. (1993). Detection of the pathogenic parasite *Toxoplasma gondii* by specific amplification of ribosomal sequences using comultiplex polymerase chain reaction. *Journal of Clinical Microbiology* **31**, 203–207.

HIGGINS, D. G., BLEASBY, A. J. & FUCHS, R. (1991). CLUSTAL V: improved software for multiple sequence alignment. *Computers and Applied Biosciences* **8**, 189–191.

LE BLANCQ, S. M., KHRAMTSOV, N. K., ZAMANI, F., UPTON, S. J. & WU, T. W. (1997). Ribosomal RNA gene organisation in *Cryptosporidium parvum*. *Molecular and Biochemical Parasitology* **90**, 463–478.

MORGAN, U. M., CONSTANTINE, C. C., O'DONOGHUE, P., MELONI, B. P., O'BRIEN, P. A. & THOMPSON, R. C. A. (1995). Molecular characterisation of *Cryptosporidium* isolates from humans and other animals using RAPD (Random Amplified Polymorphic DNA) analysis. *American Journal of Tropical Medicine and Hygiene* **52**, 559–564.

MORGAN, U. M., CONSTANTINE, C. C., FORBES, D. A. & THOMPSON, R. C. A. (1997*a*). Differentiation between human and animal isolates of *Cryptosporidium parvum* using rDNA sequencing and direct PCR analysis. *Journal of Parasitology* **83**, 825–830.

MORGAN, U. M., CONSTANTINE, C. C. & THOMPSON, R. C. A. (1997*b*). Is *Cryptosporidium* clonal? *Parasitology Today* **13**, 488.

MORGAN, U. M., SARGENT, K. D., DEPLAZES, P., FORBES, D. A., SPANO, F., HERTZBERG, H., ELLIOT, A. & THOMPSON, R. C. A. (1998*a*). Molecular characterisation of *Cryptosporidium* from various hosts. *Parasitology* **117**, 31–37.

MORGAN, U. M., PALLANT, L., DWYER, B., FORBES, D. A. & THOMPSON, R. C. A. (1998*b*). Comparison of PCR and microscopy for the detection of *Cryptosporidium parvum* in human fecal specimens – clinical trial. *Journal of Clinical Microbiology* **36**, 995–998.

MORGAN, U. M., SARGENT, K. D., ELLIOT, A. & THOMPSON, R. C. A. (1998*c*). *Cryptosporidium* in cats – additional

evidence for *C. felis*. *The Veterinary Journal* (in the Press).

MORGAN, U. M. & THOMPSON, R. C. A. (1998). PCR detection of *Cryptosporidium*: the way forward? *Parasitology Today* **14**, 241–245.

PAYNE, S. & ELLIS, J. (1996). Detection of *Neospora caninum* DNA by the polymerase chain reaction. *International Journal for Parasitology* **26**, 347–351.

PENG, M. M., XIAO, L., FREEMAN, A. R., ARROWOOD, M. J., ESCALANTE, A. A., WELTMAN, A. C., ONG, C. S. L., MacKENZIE, W. R., LAL, A. A. & BEARD, C. B. (1997). Genetic polymorphism among *Cryptosporidium parvum* isolates: evidence of two distinct human transmission cycles. *Emerging Infectious Diseases* **3**, 567–573.

SARGENT, K. D., MORGAN, U. M., ELLIOT, A. & THOMPSON, R. C. A. (1998). Morphological and genetic characterisation of *Cryptosporidium* oocysts from domestic cats. *Veterinary Parasitology* (in the Press).

SCHLOTTERER, C., HAUSER, M. T., VON HAESELER, A. & TAUTZ, D. (1994). Comparative evolutionary analysis of rDNA ITS regions in Drosophila. *Molecular and Biological Evolution* **11**, 513–522.

SPANO, F., PUTIGNANI, L., CRISANTI, A., SALLICANDO, P., MORGAN, U. M., LE BLANCQ, S. M., TCHACK, L., TZIPORI, S. & WIDMER, G. (1998). A multilocus genotypic analysis of *Cryptosporidium parvum* from different hosts and geographical origin. *Journal of Clinical Microbiology* (in the Press).

SPANO, F., PUTIGNANI, L., McLAUGHLIN, J., CASEMORE, D. P. & CRISANTI, A. (1997). PCR–RFLP analysis of the *Cryptosporidium* oocyst wall protein gene discriminates between *C. wrairi* and *C. parvum*, and between *C. parvum* isolates of human and animal origin. *FEMS Microbiology Letters* **150**, 209–217.

VAN DE PEER, V., VAN DEN BREOCK, I., DE RIJK, P. & DE WACHTER, R. (1994). Database on the structure of small subunit RNA. *Nucleic Acids Research* **22**, 3488–3494.

VASQUEZ, J. R., GOOZE, L., KIM, K., GUT, J., PATERSEN, C. & NELSON, R. G. (1996). Potential antifolate resistance determinants and genotypic variation in the bifunctional reductase-thymidylate synthase gene from human and bovine isolates of *Cryptosporidium parvum*. *Molecular and Biochemical Parasitology* **79**, 153–165.

ZHU, G., MARCHEWKA, M. J. & KEITHLY, J. S. (1998). Characterisation and phylogenetic analysis of ribosomal RNA genes in *Cryptosporidium parvum*. Unpublished (Genebank accession number AF040725).