Molecular characterization of *Cryptosporidium* from various hosts

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SUMMARY

A 298 bp region of the *Cryptosporidium parvum* 18S rDNA and a 390 bp region of the acetyl-CoA synthetase gene were sequenced for a range of human and animal isolates of *Cryptosporidium* from different geographical areas. A distinct genotype is common to isolates from cattle, sheep and goats and also an alpaca from Peru and is referred to here as the 'calf'-derived *Cryptosporidium* genotype. Another genotype of 'human'-derived isolates also appears to be conserved amongst human isolates although humans are also susceptible to infection with the 'calf' *Cryptosporidium* genotype. Mice and pigs carry genetically distinct genotypes of *Cryptosporidium*. Three snake isolates were also analysed, 2 of which exhibited *C. muris* genotypes and the third snake isolate carried a distinct 'mouse' genotype.

Key words: *Cryptosporidium*, characterization, genotypes.

INTRODUCTION

At present, 8 species of *Cryptosporidium* are considered to be valid (Fayer, Speer & Dubey, 1997). The causative agent of cryptosporidiosis in humans and other mammals is the species *C. parvum*. While few differences have been found in the morphological characteristics and developmental cycles of *C. parvum* isolates from mammalian hosts (Current & Reese, 1986), there is increasing evidence which suggests that *C. parvum* is not a uniform species. Isolate (or strain) variation has been observed for several biological and molecular characters, and may also be reflected in variable symptomatology, infectivity and virulence (Current & Reese, 1986; Fayer & Ungar, 1986; Pozio *et al*. 1992; Morgan *et al*. 1995, 1997*a*). In addition, variable responses to treatment in patients infected with *Cryptosporidium* suggests that strain variation may be a factor in the clinical management of cryptosporidiosis (Anon, 1996).

The current classification of *Cryptosporidium* species affecting mammals does not appear to reflect biological reality, and this is likely to be a limitation in epidemiological investigations. There is therefore a need for population-level information on the molecular characterization of isolates of *C. parvum* and non-*C. parvum* groups. Molecular characterization has not been possible until recently, because of the inability to amplify isolates in *in vitro* culture. The establishment of a range of PCR-based techniques and the possibility to obtain sequence data from a number of genetic loci (Morgan & Thompson, 1998) have provided the opportunity for the comprehensive molecular characterization of *Cryptosporidium* isolates. In this study, we report on a comparative analysis of genetic variation across different molecular characters among isolates of *Cryptosporidium* from a range of mammalian host species from various geographical locations.

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MATERIALS AND METHODS

Sources of parasite isolates, DNA purification and primer design

Sources of parasite isolates are listed in Table 1 and DNA was purified as previously described (Morgan *et al*. 1998). Representative samples of human and cattle isolates previously sequenced were also included (Morgan *et al*. 1997*a*). Primers used to amplify a 298 bp portion of the small subunit (SSU) ribosomal DNA were as previously described (Morgan *et al*. 1997*a*). Sequence information for the *Cryptosporidium* acetyl-CoA synthetase gene was

retrieved from GeneBank (accession no: U24082) (Khramtsov *et al*. 1996). AcetylCo A Synthetase primers designated AcoAF1 and AcoAR1 were designed using Amplify 2·1 (Bill Engels, University of Wisconsin), and oligonucleotides were synthesized by Gibco BRL (Gaithersburg, MD, USA).

PCR amplification and sequencing

Amplification conditions used to amplify a 298 bp portion of the 18S-rDNA were as previously described (Morgan *et al*. 1997*a*). The primers and their sequences used to amplify a 390 bp product from the acetyl-CoA synthetase gene were ACoAF1, forward primer (858–880) (5′ GGACCTATTGAATTTG-TCAAGG 3′) and ACoAR1, reverse primer (1227–1248) (5′ GAGTAATTCTGTGTCTCTCCAC 3′). PCR amplification was performed in 25 μl volumes with the final mix containing 0·01–1 ng of DNA, and was amplified in 67 mM Tris–HCl (pH 7·6), 16·6 mM $(NH_4)_2SO_4$, 1·5 mM $MgCl_2$, 200 μM of each dNTP, 12·6 pmoles of each primer, 0·5 units of *Tth* Plus (Biotech International, Perth, Western Australia). Reactions were performed on a PE 2400 (Perkin Elmer, Foster City, California) thermal cycler. Samples were heated to 96 °C for 2 min, followed by 45 cycles of 94 °C for 30 sec, 59 °C for 30 sec and 72 °C for 30 sec and 1 cycle of 72 °C for 7 min. PCR products were purified using Qiagen spin columns (Qiagen, Hilden, Germany), and sequenced using an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions except that the annealing temperature was raised to 58 °C. PCR products were sequenced in both directions. Sequences were analysed using SeqEd v1.0.3. (Applied Biosystems) and aligned using the Clustal V (Higgins, Bleasby & Fuchs, 1991) sequence alignment program.

Phylogenetic analysis

Phylogenetic analysis 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 (Felsenstein, 1989).

RESULTS

Sequence analysis of 18S rDNA

All the isolates listed in Table 1 from which DNA was derived and sequenced as part of this study, had oocysts with dimensions which conformed to those described for *C. parvum* (Upton & Current, 1985), apart from snake 1 and snake 2 isolates from a taipan which had oocysts corresponding to those described for *C. serpentis* (Tilley, Upton & Freed, 1990).

Sequence analysis of the 298 bp SSU-rDNA product (Fig. 1), revealed the 'calf' genotype (Morgan *et al*. 1997*a*, 1998) to be conserved between the 5 calf isolates from Australia and Switzerland. Sheep and goat isolates from Australia, Spain and Cyprus and an alpaca isolate from Peru also displayed the 'calf' genotype. Human isolates from Australia and the UK exhibited the 'human' genotype with the exception of 1 human isolate (H18) which displayed the 'calf' genotype. Sequence analysis of pig isolates from Switzerland and Australia revealed that pigs harbour a distinct genotype of *Cryptosporidium*. Isolates of *Cryptosporidium* from mice were also genetically distinct. Two of the snake isolates harboured *C. muris* and the third snake isolate carried the 'mouse' genotype. Representative human (P12), calf (C1), pig (Pig 1) and mice (M7) sequences are aligned in Fig. 1.

Sequence analysis of the acetyl-CoA synthethase gene

As with the rDNA sequencing results, sequence analysis of the acetyl-CoA synthetase gene revealed distinct differences between human and animal isolates (Fig. 2). All human isolates analysed exhibited a common genotype with the exception of H18 which displayed the 'calf' genotype. The calf, sheep and goat isolates and the alpaca isolate were genotypically identical. A total of 5 mouse isolates were sequenced using the acetyl-CoA primers and all displayed a genetically distinct genotype. The third snake isolate which displayed the 'mouse' genotype using rDNA sequence analysis also exhibited this genotype using acetyl-CoA sequence analysis. Snake isolates 1 and 2 did not amplify using these primers. Only 1 pig isolate amplified using the acetyl-CoA primers (Pig 2) and this isolate displayed the same genotype as that of *Cryptosporidium* from cattle. Representative human (P12), calf (C1) and mouse (M7) sequences are aligned in Fig. 2. The reference isolate (KSU-1) from which the original acetyl-CoA synthetase gene sequence information was derived (Khramtsov *et al*. 1996), was isolated from a calf and was included for comparison (Fig. 2).

Phylogenetic analysis of rDNA sequencing results

Additional isolates previously sequenced were also analysed and included a koala isolate (K1) (Morgan *et al*. 1997*a*), domestic cat isolates (Sargent *et al*. 1998), and additional *Cryptosporidium* and coccidian (*Toxoplasma gondii* and *Neospora caninum*) isolates retrieved from the rRNA WWW server (http://rrna.uia.ac.be/) (van de Peer *et al*. 1994). This extended phylogenetic analysis resulted in 5 distinct

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; IP, Institute of Parasitology, Zurich, Switzerland; MA, Ministry of Agriculture, Department of Veterinary Services, Nicosia, Cyprus; MU, Murdoch University, Western Australia; PMH, Princess Margaret Hospital, Perth, Western Australia; SHL, State Health Laboratories, Perth, Western Australia.)

Code	Host	Geographic origin	Source
H1	Human	Perth, WA	PMH
H7	Human	Perth, WA	SHL
H16	Human	Perth, WA	PMH
H18	Human	Perth, WA	PMH
P12	Human	Wales	IDR
P18	Human	Central England	IDR
P29	Human	South England	IDR
A1	Alpaca	Peru	IDR
S1	Sheep	Spain	IDR
M7	Mouse	Victoria	CSIRO
M11	Mouse	Walpeup, Victoria	CSIRO
M24	Mouse	Walpeup, Victoria	CSIRO
M26	Mouse	Victoria	CSIRO
M27	Mouse	Walpeup, Victoria	CSIRO
C1	Calf	Millicent, SA	CVL
sC11	Calf	Switzerland	IP
sC26	Calf	Switzerland	IP
sC27	Calf	Switzerland	IP
sC33	Calf	Switzerland	IP
G1	Goat	Toodyay, WA	AgWA
G2	Goat	Cyprus	MA
G3	Goat	Cyprus	MA
G4	Goat	Cyprus	MA
Pig 1	Pig	Switzerland	IP
Pig 2	Pig	Popanyinning, WA	MU
Pig 3	Pig	Popanyinning, WA	MU
Pig 4	Pig	Popanyinning, WA	MU
Snake 1	Taipan	Tanunda SA	CVL
Snake 2	Taipan	Tanunda SA	CVL
Snake 3	Woma Python	Perth Zoo, WA	MU

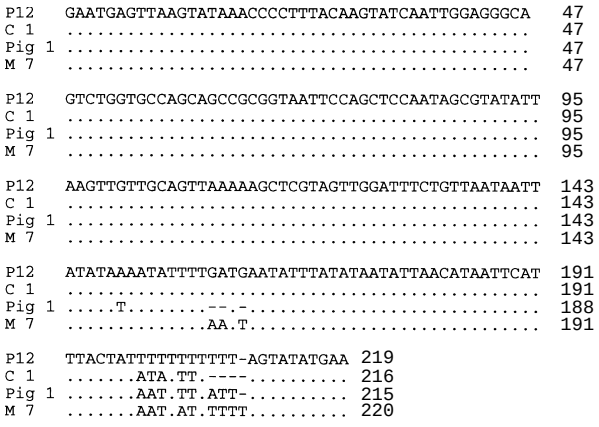

Fig. 1. Sequence alignments of the rDNA PCR fragment from representative human (P12), calf (C 1), pig (Pig 1) and mouse (M 7) *Cryptosporidium parvum* isolates.

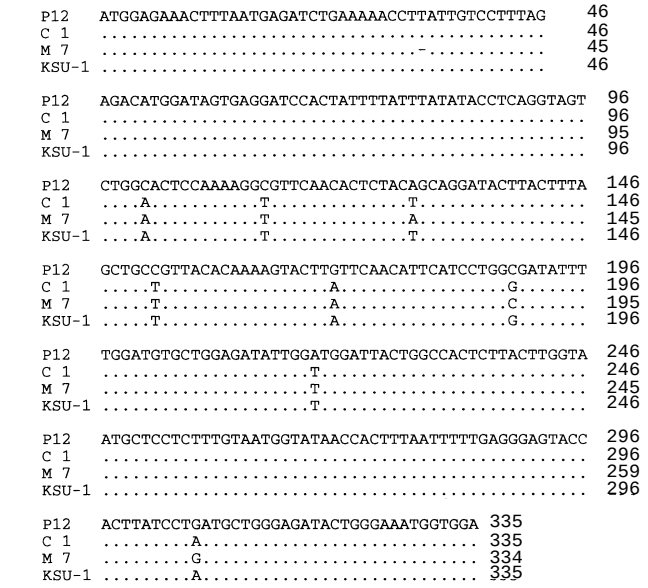

Fig. 2. Sequence alignments of the acetyl-CoA synthethase gene PCR fragment from representative human (P 12), calf (C 1) and mouse (M 7) *Cryptosporidium parvum* isolates (KSU-1 = reference sequence from Kramstov *et al*. 1996).

groups within *C. parvum* (Fig. 3). A 'human' group, a 'calf' group which contained most of the animal isolates and 1 human isolate (H18); the koala isolate was genetically distinct as were the pig isolates which were grouped separately and exhibited a distinct genotype from that found in cattle and other livestock. The pig genotype appeared to be conserved as the pig isolate from Switzerland (Pig 1) and the pig isolates from Australia (Pig 2, Pig 3 and Pig 4) were identical. The isolates of *C. parvum* from mice were also grouped separately, and were very different from *C. muris*, being most closely related to *Cryptosporidium* isolated from pigs. The cat isolates have previously been shown to be genetically very distinct from all other *Cryptosporidium* isolates (Sargent *et al*. 1998). In addition, *Cryptosporidium* oocysts identified in faeces from these cats were smaller than isolates seen in humans. Oocysts from cat samples had a mean size of $4{\cdot}6 \times 4{\cdot}0\ \mu$m, while those from humans averaged $5{\cdot}0 \times 4{\cdot}5\ \mu$m, 4 additional cat isolates were sequenced which were identical to the previous cat sequences (Morgan *et al*. unpublished observations). These results lend additional support to the concept of a cat-specific strain or species of *Cryptosporidium*.

Phylogenetic analysis of acetyl-CoA synthetase gene sequencing results

Phylogenetic analysis of the acetyl-CoA synthetase gene sequence information produced 3 groups (Fig. 4). A 'human' group, a 'calf' group which contained most of the animal isolates as well as the H18 human isolate which was grouped with the animal isolates using rDNA analysis. A total of 5 isolates from mice

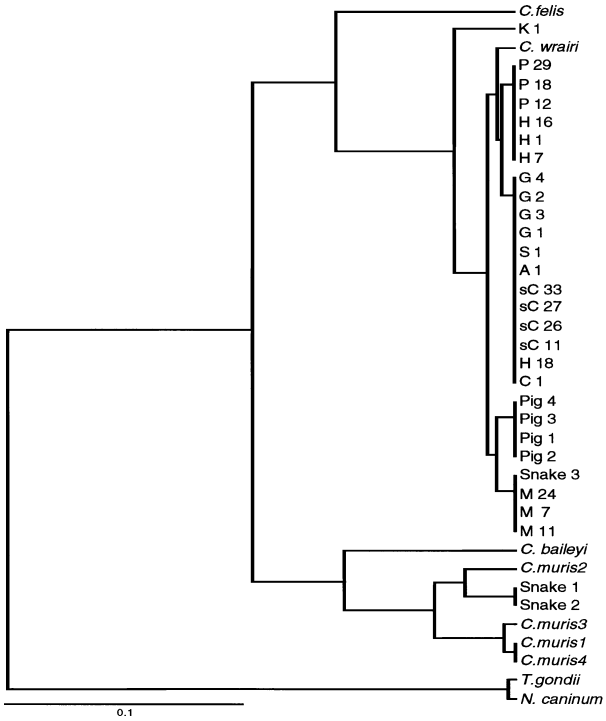

Fig. 3. Extended phylogram of Kimura's distance generated from 18S rDNA sequence information amongst isolates of *Cryptosporidium* clustered using the Unweighted Pair Group Method of Analysis (UPGMA). (Includes additional isolates previously sequenced such as a koala (K 1) isolate (Morgan *et al*. 1997*a*), cat isolates (Sargent *et al*. 1998), and additional *Cryptosporidium* and coccidian isolates retrieved from the rRNA WWW server (http://rrna.uia.ac.be/) (van de Peer *et al*. 1994).

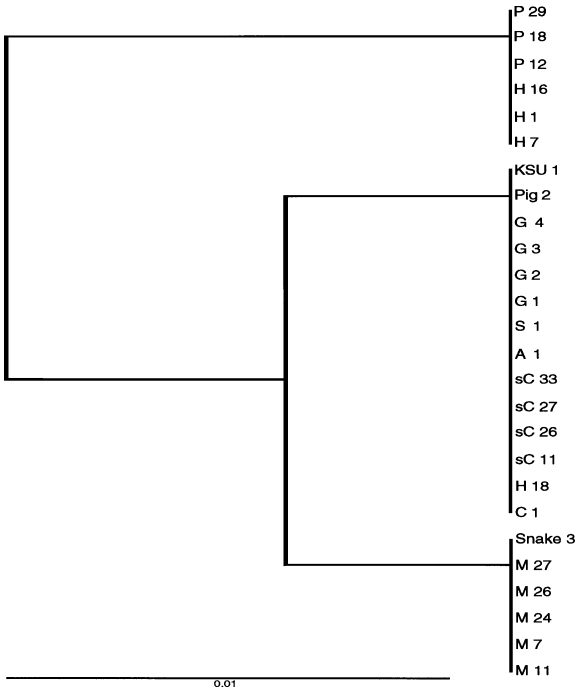

Fig. 4. Phylogram of Kimura's distance generated from acetyl-CoA synthethase gene sequence information amongst isolates of *Cryptosporidium* clustered using UPGMA.

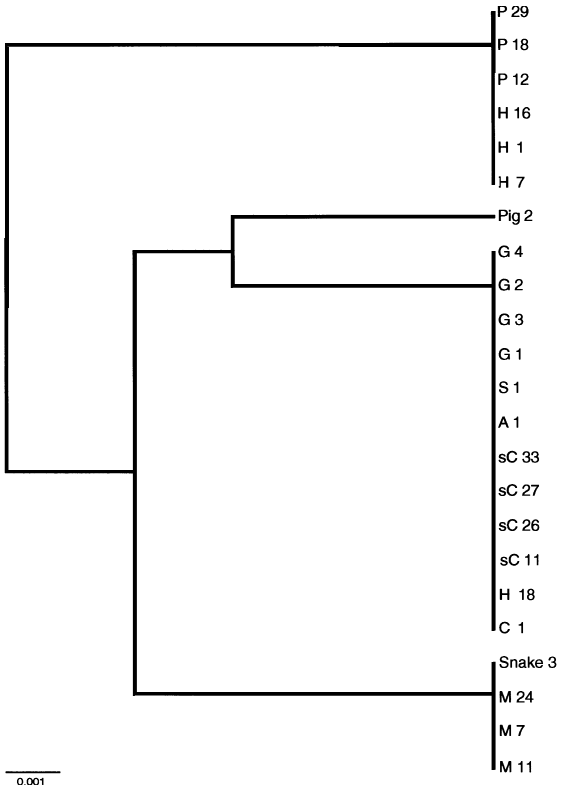

Fig. 5. Phylogram of Kimura's distance generated from the combined rDNA and acetyl-CoA synthethase gene sequence information amongst isolates *Cryptosporidium* clustered using UPGMA.

were sequenced using the acetyl-CoA primers all of which formed a distinct group on their own. Only 1 pig isolate (Pig 2) amplified using the acetyl-CoA primers and sequence analysis of this PCR product aligned it with the 'calf' genotype.

Phylogenetic analysis of combined rDNA and acetyl-CoA synthetase gene sequencing results

Combined analysis of the rDNA and acetyl-CoA sequences resulted in 4 major groups; 'calf', 'human', 'pig' and 'mouse' (Fig. 5). As sequence information could only be obtained from the pig isolate Pig 2, and the snake isolate (snake 3), all the other pig, snake and koala isolates were excluded from the combined analysis.

DISCUSSION

In this study, both 18S rDNA and acetyl-CoA synthetase gene sequence analysis has confirmed the widespread distribution of a distinct genotype of *C. parvum* (referred to here as the 'calf' genotype) common to domestic livestock including cattle, sheep, goats and an alpaca from Peru. The calf genotype was also isolated from humans. However, another genotype (the 'human' genotype) appears to be conserved amongst human isolates, and may be host specific. Pigs and mice exhibited distinct

genotypes and a number of snake isolates were found to exhibit *C. muris* and 'mouse' genotypes respectively.

Sequencing a protein-coding gene such as the acetyl-CoA synthetase gene has provided both a useful comparison with 18S rDNA sequencing and a valuable molecular epidemiological tool for characterizing *Cryptosporidium* isolates from different hosts. Sequence analysis of the *Cryptosporidium* dihydrofolate reductase–thymidylate synthase has revealed differences between a human and a calf isolate (Vasquez *et al*. 1996). In the present study, sequence analysis of the conserved acetyl-CoA synthetase coding gene also confirmed the genetic distinctness of the 'human' and 'calf' genotypes of *C. parvum*, with the mouse isolates also exhibiting a distinct genotype. Interestingly, the pig isolates were shown by rDNA sequence analysis to be genetically distinct, whereas acetyl-CoA synthetase sequence analysis of one of these isolates grouped it with isolates from cattle. Although there is a need for the molecular characterization of additional isolates of *Cryptosporidium* from pigs, the present results are most likely due to the more conserved nature of the acetyl-CoA coding region.

Cryptosporidium has been reported to be the most frequently isolated pathogen in goat kids with gastrointestinal disease (Nagy *et al*. 1984). In northern Spain, 70% of kids sampled were infected and all the farms visited had cryptosporidiosis in their flocks (Matos-Fernandez *et al*. 1994). In the present study, goat isolates from both Australia and Cyprus all exhibited the 'calf' genotype. The isolate from Australia (G1) was from an outbreak of diarrhoea in 1 to 2-week-old goats. The isolates from Cyprus (G2–G4), were asymptomatic cases that were collected at random.

Previous analysis by Spano *et al*. (1997) of the sheep isolate (S1) and the alpaca isolate (A1) using RFLP analysis of the *Cryptosporidium* oocyst wall protein gene (COWP) also showed that these isolates belonged to the 'calf' group. rDNA and acetyl-CoA sequencing of the human isolates P12, P18 and P29 from the UK confirmed their 'human' genotype grouping (Spano *et al*. 1997) and their identity with isolates from humans in Australia indicates that the 'human' genotype is very conserved. One human isolate (H18), had previously been shown to display the 'calf' genotype (Morgan *et al*. 1997*a*). Research in our laboratory, screening large numbers of faecal samples, has shown that approximately 17% of isolates of *Cryptosporidium* infecting humans display the 'calf' genotype (Morgan *et al*. 1998), indicating the potential for zoonotic transmission. In the present study, we have demonstrated the widespread distribution of the 'calf' genotype in cattle, sheep, goats and an alpaca which further emphasizes the role of domestic livestock as important potential zoonotic reservoirs of human cryptosporidial infection. The broad distribution of the 'calf' and 'human' genotypes, and probably also the 'pig', lends further support to the concept of a clonal population structure for *Cryptosporidium* (Morgan, Constantine & Thompson, 1997*b*).

Little is known of the prevalence of any species of *Cryptosporidium* in pigs (Fayer, Speer & Dubey, 1997). This is the first time that pig isolates have been analysed genetically and our results using rDNA sequence analysis indicate that pigs carry a host-adapted genotype of *C. parvum* that is conserved across wide geographical areas. The pig isolate from Switzerland (Pig 1) was from an asymptomatic infection whereas the Western Australian isolates (Pig 3, Pig 3 and Pig 4) were from 26-day-old pigs which died in a commercial piggery.

Mice (*Mus musculus*) from different agricultural areas in Victoria, were shown to carry a distinct genotype of *C. parvum* using both rDNA and acetyl-CoA synthetase sequence analyses. In total 182 mice from various locations were screened, 11 of which were positive for *C. parvum* (a prevalence of 6%) but *C. muris* was not detected. This is the first time that isolates of *C. parvum* from mice have been analysed genetically. Previous studies on wild brown rats (*Rattus norvegicus*) in the UK reported a 63% ($n = 73$) prevalence of *C. parvum* and again *C. muris* was not detected (Webster & MacDonald, 1995). A more recent 2-year study at a farm in Warwickshire, UK on wild mice and voles reported prevalence figures of 22, 21 and 13% for *C. parvum* in *M. musculus* syn. *domesticus*, *Apodemus sylvaticus* and *Clethrionomys glareolus*, respectively, figures higher than those reported for *C. muris* at 10, 6 and 2% (Chalmers *et al*. 1997). The apparent autumnal peak for *C. parvum* in all 3 rodent species coincided with the calving period at the farm and it was concluded that 'rodents may represent a significant reservoir of *Cryptosporidium* with a high potential for infection of man and livestock due to cohabitation' (Chalmers *et al*. 1997). However given the genetic distinctness of the *C. parvum* isolates from wild mice examined in the present study, mice may not play a role in the transmission of *Cryptosporidium* from animals to humans. Clearly, more extensive analysis of rodent isolates from a wider geographical distribution is necessary before their role as reservoirs of infection in humans and domestic animals can be fully determined.

Cryptosporidium infections have been reported in over 57 different reptilian species (O'Donoghue, 1995). At present, *C. serpentis* is the only recognized species described in snakes (Fayer *et al*. 1997). However, morphometric studies on isolates recovered from snakes and lizards have indicated the occurrence of at least 5 different morphological types (Upton *et al*. 1989). Two of the snake isolates in this study (Snakes 1 and 2) were from chronically, heavily infected adult taipans (*Oxyuranus scutellatus*)

isolated by the Central Veterinary Laboratories (CVL) in Adelaide, South Australia, where they had been diagnosed as *C. serpentis* on the basis of host occurrence and also morphometrics (the dimensions of the oocysts of these 2 isolates were 6·2 × 5·4 μm and 6·3 × 5·5 μm respectively, Dr P. O'Donoghue, personal communication). The snake 3 isolate was from a Woma Python (*Aspidites ramsayi*) from Perth Zoo in Western Australia (oocysts approximately 4·5 × 4·0 μm). The 18S sequence analysis of these isolates revealed the first 2 isolates to be *C. muris* and the third to display the 'mouse' genotype. Only the snake 3 isolate amplified using the acetyl-CoA primers and sequence analysis of this PCR product also confirmed it had the 'mouse' genotype. The fact that the 'mouse' genotype has also been identified in Western Australia, confirms the widespread distribution of this genotype. It is possible that the snakes were not actually infected but simply passing oocysts from ingested mice that were infected with *Cryptosporidium*. However, this is unlikely as infections in all 3 snakes were heavy and in the case of the first 2 snakes, large numbers of oocysts were passed over an extended period of time. There is considerable variation in the dimensions of *C. muris* oocysts which can vary in length from 6·6 to 7·9 μm and from 5·3 to 6·5 μm in width (Upton & Current, 1985). The oocysts diagnosed by CVL were at the lower limit of the size range for *C. muris* at 6·3 × 5·5 μm and this highlights the importance of genotyping isolates for the purposes of speciation. Tilley *et al.* (1990), have compared oocysts from a naturally infected rat snake (*Elaphe obsoleta quadrivittata*) with those of *C. parvum* using protein electrophoresis. In their study, oocysts from snakes measured approximately 6·2 × 5·3 μm, were not infective to mice and displayed a distinctly different protein profile to oocysts from *C. parvum*. However, the snake isolate was not compared with other isolates and species of *Cryptosporidium* such as *C. muris*. It is possible that snakes are capable of being infected with whatever genotype they ingest and that *C. serpentis* does not exist as a separate species, however, a wide range of isolates from reptiles from different geographical areas needs to be examined before any such conclusions can be made.

The results of this study further demonstrate that *C. parvum* can not be considered to be a uniform species. A series of host-adapted genotypes exist and on the basis of the phylogenetic analysis undertaken in this study, some or all of these genotypes may represent distinct species. This is highlighted by the much greater genetic differences found between genotypes of *Cryptosporidium* than between the genera *Toxoplasma* and *Neospora*. In our view, the present study emphasizes the need for a taxonomic revision of the genus *Cryptosporidium* although this will require additional comparative studies. As with any disease-causing organism, an evolutionarily sound classification and accurate understanding of the host range and zoonotic potential of *Cryptosporidium* is essential for successful public health programmes. The evidence presented here and in other studies (Awad-El-Kariem *et al.* 1995; Morgan *et al.* 1995, 1997*a*, 1998; Peng *et al.* 1997; Spano *et al.* 1997; Vasquez *et al.* 1996) indicates that the 'calf' and 'human' genotypes are very common and geographically widespread. Further, only the 'human' and 'calf' genotypes appear on present evidence to be capable of infecting immunocompetent humans whereas those genotypes characterized from pigs, mice and cats may be host specific and have not so far been isolated from humans.

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