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REVIEW

Phenotypic and genotypic characterization of *Cryptosporidium* species and isolates

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Recent outbreaks of cryptosporidiosis from contaminated water supplies have led to a need for the detection of *Cryptosporidium* oocysts from various hosts and contaminating sources. The presence of nonpathogenic species or strains of *Cryptosporidium* is important for diagnostic purposes as there is a potential for false-positive detection of pathogenic parasites. The present review focuses on phenotypic differences and recent advances in genotypic analyses of the genus *Cryptosporidium* with an emphasis on detecting various isolates and identifying differences in *Cryptosporidium parvum* and other species in this genus. The information currently available demonstrates important patterns in DNA sequences of *Cryptosporidium*, and our understanding of macro- and microevolutionary patterns has increased in recent years. However, current knowledge of *Cryptosporidium* genetic diversity is far from complete, and the large amount of both phenotypic and genotypic data has led to problems in our understanding of the systematics of this genus. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 95–106.

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Introduction

Species of the genus *Cryptosporidium* are apicomplexan protozoa that are pathogenic in various hosts (Table 1). Of particular importance to humans is *Cryptosporidium parvum* [25]. Infections with this parasite can cause various symptoms associated with gastrointestinal infections, and immunocompromised hosts are particularly susceptible. Infections can result by ingestion of infective oocysts through unhygienic conditions and contaminated food and water. In recent years, there have been several outbreaks of cryptosporidiosis resulting from contaminated municipal water supplies. The parasite has become a high research priority for detection and inactivation from environmental sources such as water.

The life cycle of *C. parvum* is similar to those of other *Cryptosporidium* spp. [25,26,62]. It has a one-host life cycle and hosts become infected by ingestion of oocysts in water or other contaminated substances. Life-cycle stages occur on the surface of the microvilli, and begin with sporozoites emerging from the oocyst and infecting epithelial cells. Various asexual stages of merogony and gametogony occur and resemble those of the coccidia. Fusion of microgametes and macrogametes results in the formation of a zygote that undergoes sporogony, and the final sporulated oocyst contains four sporozoites. The sporulated oocysts are excreted in the feces and can remain infective in the environment for extended periods.

In addition to *C. parvum*, there has been an increased interest in the systematics of other species in the genus for several reasons. First, oocysts of other *Cryptosporidium* species may occur in water and it would be beneficial to the water works industry to be able to distinguish various species and strains within the genus. Such diagnostic methods may lead to development of protocols to allow the determination of contaminant sources. Second, other, less pathogenic species are important because their presence may lead to false-positive diagnoses for *C. parvum*. Third, the recent identification of *Cryptosporidium felis* in a cow [14] and in humans [56,68], as well as the detection in humans of an isolate resembling *Cryptosporidium meleagridis* from birds [56], has indicated the possibility that some species distinct from *C. parvum* may infect a wider variety of hosts than has previously been thought.

Detection methods based on recovering oocysts from water samples and identifying *Cryptosporidium* by microscopic techniques are well described [1,2,28]. Current methods of detection involve filtering oocysts from water samples and staining them with fluorescent dyes or fluorescent conjugated antibodies. Although these methods are capable of detecting *Cryptosporidium* species, they are time-consuming, expensive, and usually nonspecific. An alternative detection strategy is the development of PCR-based methods to amplify *Cryptosporidium*-specific DNA or RNA sequences [103]. These methods are effective in identifying *Cryptosporidium* in environmental samples and they continue to be improved for use as a suitable detection tool. Another potential benefit of PCR technology is the use of DNA sequences of *Cryptosporidium* species that can provide significant genotypic information. This information is important in determining the sources of *Cryptosporidium* in the environment and may eventually provide information on relative pathogenicity of various isolates of *C. parvum* and other species of *Cryptosporidium*.

In this review, progress on our knowledge of species and strain differences in the genus *Cryptosporidium* is presented. Several

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Table 1 Species of *Cryptosporidium* with original distinguishing features

Species	Type host	Oocyst dimensions (μm)	Distinguishing features
C. andersoni,[a] Lindsay *et al*, 2000 [42]	cattle (*Bos taurus*)	7.4×5.5	– found in abomasum; larger oocysts than *C. parvum*; not infective to mice, chickens, or goats
C. baileyi,[a] Current *et al*, 1986 [24]	domestic chicken (*Gallus domesticus*)	6.2×4.6	– oocysts larger than *C. parvum*, smaller than *C. muris*; not infective in suckling mice and goats
C. felis, Iseki, 1979 [34]	domestic cat (*Felis catis*)	5.0×4.5	– morphological: none – oocysts not infective to mice and guinea pigs
C. meleagridis, Slavin, 1955 [75]	turkey (*Meleagris gallopavo*)	4.5×4.0	– morphological: none – host specificity?
C. muris[a], Tyzzer, 1907 [89]	mouse (*Mus musculus*)	7×5	– located in gastric epithelium, oocysts larger than those of *C. parvum*
C. nasorum, Hoover *et al*, 1981 [32]	naso tang fish (*Naso lituratus*)	none provided	– morphological: none – host specificity in fishes
C. parvum,[a] Tyzzer, 1912 [91]	mouse (*Mus musculus*)	up to 4.5	– intestinal epithelium, oocysts smaller than those of *C. muris*; infects many vertebrate hosts
C. saurophilum,[a] Koudela and Modry, 1998 [36]	skinks (*Eumeces schneideri*)	5.0×4.7	– smaller oocysts than *C. serpentis*, different location in intestine, not infective to snakes
C. serpentis Levine, 1980 [39]	snakes (*Elaphe* spp., *Crotalus* spp., *Sansinia* spp.)	3.0×3.8 [15]; 6.2×5.3 [87]	– morphological: larger oocysts than *C. parvum*, although type description differs from subsequent oocyst measurements – host specificity
C. wrairi, Vetterling *et al*, 1971 [95]	guinea pig (*Cavia porcellus*)	– none reported in type description; 5.4×4.6 in Tilley *et al* [88]	– morphological: none; not infective to mice, rabbits, turkeys, and chickens

[a]Species considered to be valid based on phenotypic characters.

species have been insufficiently described by phenotypic criteria, and recent progress in analysis of DNA sequences has produced an alternative, and sometimes conflicting source of data that can be used to identify species. The recent discovery of distinct human and animal isolates of *C. parvum* using multilocus sequence analysis has led to loci that may be used to determine the origin of oocysts in water samples and the relative pathogenicity to humans of various species. These studies provide useful information on the epidemiology of various strains and species and are facilitating diagnosis of the parasites.

Taxonomic descriptions of *Cryptosporidium* species

The accepted number of valid *Cryptosporidium* species has varied over the past few decades. One of the main reasons for this variation has been the naming of distinct species based on host specificity. However, the finding that *C. parvum* can parasitize numerous mammalian hosts has led to the synonymization of many species with *C. parvum*. At present, there is evidence for at least five *Cryptosporidium* species based on original taxonomic descriptions and diagnosable differences with other species (Table 1); however, as many as 10 that have been described are considered valid by some authors (Table 1).

The first species of *Cryptosporidium* to be described was *Cryptosporidium muris* from the gastric glands of mice. The first naming of the species [89] did not include a formal description. However, details on the life cycle and taxonomy of *C. muris* were provided 3 years later [90]. Although similarities to the gregarines were noted, the organisms were classified as coccidia.

C. parvum was originally isolated from mice. The main difference between the two species was that *C. parvum* was found in the small intestine and *C. muris* in gastric cells [91]. Also, *C. muris* oocysts measured 7×5 μm whereas *C. parvum* oocysts did not exceed 4 to 4.5×3.3 μm [90,91]. For both species, a life cycle involving transmission of the parasites through contaminated feces was demonstrated.

The most recent species to be described was *Cryptosporidium andersoni*, which was reported from the abomasum of cattle in the United States. Its oocyst dimensions are similar to those of *C. muris*. However, the oocysts were not infective to mice, chickens or goats, and DNA sequence data demonstrated that the species was distinct from *C. muris* [42]. The original description included illustration of the oocysts, cross-infection data, and information regarding molecular differences between *C. andersoni* and other species.

Another species, *Cryptosporidium wrairi*, was described from the small intestine of guinea pigs. Merogonic and gametogonic descriptions of the parasites were provided but there was no description of the oocyst [95]. Ileal scrapings given to other hosts indicated that this species was not infective to mice, rabbits, chickens or turkeys. The host specificity, as well as a location in the gut that differed from *C. muris*, provided the basis for distinguishing *C. wrairi* as a separate species [95]. However, the lack of controlled cross-infection experiments as well as lack of an oocyst description provided no suitable basis for naming a distinct species of *Cryptosporidium* from guinea pigs.

More recent experiments have demonstrated that guinea pigs given oocysts of *C. parvum* from calves can serve as suitable hosts

for this species [93]. It has also been demonstrated that suckling mice have been infected successfully with a guinea pig *Cryptosporidium* isolate and that the oocysts closely resemble those of *C. parvum* [88]. These studies further challenge the distinct species status of *C. wrairi*.

The life cycle stages of another species, *C. meleagridis*, were described from the small intestine of turkeys. The parasite was almost identical to *C. parvum* as originally described by Tyzzer, but was given distinct species status, "provisionally named," presumably because of the different host [75]. The species description excluded information on the sporulated oocyst and cross-infection data, and cannot be distinguished from *C. parvum* or from several other species. More recently, *Cryptosporidium baileyi* was described from the bursa of Fabricius and the cloaca of chickens. Inoculation with oocysts into suckling mice, goats, and quail did not produce infections, although ducks and geese were infected experimentally with *C. baileyi* [24]. The oocysts (6.3×5.2 μm) are larger than those of *C. parvum* and *C. meleagridis*.

C. felis was isolated and described from the small intestine of domestic cats in Japan. Endogenous life cycle stages were described by using light and electron microscopy. The parasites from cats did not infect mice and guinea pigs, and the oocysts, which measured 5×4.5 μm, were reported to differ in size from other species excluding *C. parvum* [34]. Iseki may have assumed that *C. parvum* was only infective to mice, and the lack of infectivity in mice of *C. felis* provided the basis of differentiation from this species. More recently, Asahi *et al* [3] demonstrated that *Cryptosporidium* sp. oocysts isolated from cats were not infective to mice, rats, guinea pigs, dogs or suckling mice.

C. felis was not listed as a valid species by some authors [40,62]. However, Fayer *et al* [26] included it in a list of valid named species. Although morphological descriptions of *C. felis* closely resemble those of *C. parvum*, molecular data appear to provide evidence of a distinct species. A distinct *C. felis* genotype has been reported in humans (see below).

Several reports exist of piscine infections with *Cryptosporidium* [26]. However, the only species currently accepted by many authors is *Cryptosporidium nasorum*. Endogenous stages described from an infected naso tang (*Naso lituratus*) based on histologic and electron microscopic information provided the basis for the original species description. No data were provided on the morphology of the oocysts and the life cycle of *C. nasorum* was unknown [32]. According to article 13 of the International Code of Zoological Nomenclature [33], a species description should be accompanied by a description or definition that describes characters that differentiate the species from others. The lack of a description or definition that differentiates *C. nasorum* from other *Cryptosporidium* species renders it a *nomen nudum*.

Other reports of *Cryptosporidium* spp. in fish exist, although full morphological and life cycle descriptions are incomplete. Recently, the genus *Piscicryptosporidium* Paperna and Vilenkin 1996 was proposed to accommodate two species of parasites closely resembling *Cryptosporidium* spp. [66]. *Piscicryptosporidium* differed from *Cryptosporidium* spp. in having sporulated oocysts that sink into the lamina propria rather than remaining on the distal portion of the epithelial cells of the stomach. Other stages, however, were found above the surface of the villous border. Reports of *Cryptosporidium*-like parasites from fish are uncommon and require further investigation, particularly in determining life cycles and in molecular phylogenetic analyses. Information from piscine *Cryptosporidium* species will be vital in developing an understanding of the evolution and genetic diversity of the genus that can be compared to those species for which more data are available.

Several *Cryptosporidium* species have been isolated from reptiles and described. However, many authors currently accept only one species, *Cryptosporidium serpentis*. Several other *Cryptosporidium* species that had been previously named have since been shown to be sporocysts of *Sarcocystis* spp. [94]. Levine [39] named *C. serpentis* based on an earlier description by Brownstein *et al* [15] of a *Cryptosporidium* sp. found in three genera (*Elaphe*, *Crotalus*, and *Sansinia*) of captive snakes. He later synonymized it with *Cryptosporidium crotali* [40], which has since been shown to be identical to *Sacrocystis* sp. sporocysts [94]. Brownstein *et al* [15] reported oocysts 3 to 3.8 μm in diameter. Tilley *et al* [87] described *C. serpentis* oocysts as 6.2×5.3 μm, and in a study of numerous reptiles, Upton *et al* [94] reported oocysts larger than those described by Brownstein *et al* [15]. The larger oocyst dimensions are currently accepted as valid despite the fact that they do not correspond to the dimensions given originally by Brownstein *et al* [15].

Naming a new species by referring to another published and incomplete description is taxonomically unacceptable and *C. serpentis* is a *nomen nudum* according to the International Code of Zoological Nomenclature [33]. Given the variation in oocyst morphology in isolates from reptiles [94], it is likely that several other species from reptiles exist. Although oocysts of *Cryptosporidium* spp. from snakes appear to be infective only for snakes [30,87], several species may have similar general properties. The species *C. serpentis* is currently used as a term of convenience that likely includes several distinct species of *Cryptosporidium* from reptiles. However, because of the incomplete original species description including detailed oocyst descriptions and other comparative diagnostic information, *C. serpentis* has never been formally described. Type specimens should be described for any new species. Life cycles and isolates should also be established before new species are named [94].

The diversity of different reptilian hosts and widespread localities will likely lead to the description of other *Cryptosporidium* species in snakes and other reptiles. Recently *Cryptosporidium saurophilum* was described from lizards. The oocysts of this species measure 5.0×4.7 μm, are found in the intestine and cloaca, and are not infective for snakes [36]. Isolation of oocysts from similar hosts, study of endogenous stages, and experimental infections in other hosts may reveal the presence of other species in lizards.

Numerous other *Cryptosporidium* species have been described from different hosts. However, biological information for several of the species described above has led to an overall consensus on their validity even though descriptions and life cycle data are incomplete for some of them. There is need for a detailed redescription of *C. serpentis* and *C. nasorum*, and the criteria described for distinguishing *C. meleagridis* and *C. wrairi* should be more thoroughly investigated by cross-transmission experiments as well as molecular analyses (see below) with the objective of providing reliable species identification.

A recent set of cross-transmission experiments was reported by Bekesi *et al* [9] who suggested that *C. baileyi*, *C. muris* and *C. parvum* are unable to establish in fish, amphibians or reptiles based on experimental infections of carp, turtles, and frogs. Similarly, it was shown in another study that rat snakes experimentally inoculated with oocysts of *C. muris*, *C. wrairi*, *C. baileyi*, and *C. meleagridis* did not become infected although oocysts from other reptiles such as turtles and lizards were infective to the rat snakes

[30]. These cross-infection studies provide some evidence of the evolution of more than one *Cryptosporidium* species and they complement recent molecular phylogenetic analyses (see below).

The infectivity of an isolate of *C. muris* (strain RN 66) has also been tested in other hosts [35]. The host species most susceptible to this strain were mice and cats. Guinea pigs, rabbits and dogs showed a lower susceptibility to the *C. muris* strain [35]. Despite experiments such as these, cross-infection studies, as well as detailed morphological and life cycle information for all of the various *Cryptosporidium* species are incomplete, making a robust taxonomic scheme for this genus based on phenotypic characters confusing and without consensus.

Beyond oocyst morphology and cross-infection — molecular systematics of *Cryptosporidium*

Since the 1980s, when it was discovered that *Cryptosporidium* spp. can be pathogenic for humans, especially in immunocompromised hosts such as AIDS patients, there has been an increased interest in the biology of these organisms. This led to the development of methods to distinguish species that infect humans from other *Cryptosporidium* species and apicomplexan parasites. During the mid-to-late 1980s, it was discovered that the small subunit ribosomal RNA (SSU rRNA) gene sequences had several properties that render them useful in phylogenetic analysis [31,64]. The SSU rRNA genes are of an intermediate size relative to the large subunit (LSU) rRNA and 5.8S rRNA genes, and are among the slowest evolving sequences found throughout living organisms. There is enough evolutionary information in these sequences to allow for measurement of both close and distant phylogenetic relationships [31,64,76]. The database for these sequences is rapidly growing, and our knowledge of apicomplexan evolution has been influenced considerably from phylogenetic analyses of SSU rRNA gene sequences.

What is *Cryptosporidium*?

Molecular phylogenetic analyses of the Apicomplexa that have included *Cryptosporidium* have consistently grouped *Cryptosporidium* species as a clade separate from the coccidian taxa with which they are presently classified [8,18,21,44,59,70,86]. This pattern contradicts the presumed classification of *Cryptosporidium* within the coccidia based on similar life cycle features [41].

In recent phylogenetic analyses of gregarine parasites of insects, it was found that the gregarines from a monophyletic clade that is a sister group to *Cryptosporidium* spp. [22]. The gregarine/*Cryptosporidium* clade was separate from another major apicomplexan clade containing the coccidia, adeleids, piroplasms and haemosporinids, indicating that *Cryptosporidium* has a closer phylogenetic association with the gregarines than the coccidia. These studies were the first analyses of apicomplexans that included gregarine species. They demonstrate the need for more inclusive phylogenetic analyses that should incorporate other apicomplexans such as the protococcidians, adeleids, and other gregarines. Such studies will eventually clarify the position of *Cryptosporidium* within the phylum Apicomplexa.

The findings described above that *Cryptosporidium* may not be a coccidian are important as there is currently no effective treatment for cryptosporidiosis [10], and development of cryptosporidicidal agents based on anticoccidial agents may not be effective as the two groups of parasites are more distantly related than has been thought previously. The distant phylogenetic affinities may also be important in assessing detection methods for *Cryptosporidium* species.

Phylogenetic relationships within the genus

In recent years, SSU rRNA sequences from various *Cryptosporidium* species and isolates have been used in inferring phylogenetic relationships within the genus. The sequences have been used primarily in searching for evidence of distinct species. The accumulation of new molecular data is beginning to provide more information on the evolution of the genus. However, interpretation of molecular data has also been problematic. The degree of sequence divergence has been difficult to interpret in a taxonomic context and lack of thorough and consistent phylogenetic methodology has resulted in varying interpretations of the evolution and taxonomy of *Cryptosporidium* species.

There are no criteria for determining the number of sequence differences required to separate species. In an analysis of several *Cryptosporidium* species [92], it was determined that the genetic variation at both intra- and interspecies levels was insufficient to distinguish different species of *Cryptosporidium*. Based on SSU rRNA gene sequence differences, it was argued that with the possible exception of *C. muris*, there was insufficient nucleotide variation to provide support for different species. There appeared to be greater variation in the sequences of several *Plasmodium* species. These differences would apparently provide stronger support for distinct species status in the *Plasmodium* species [92].

The comparison between *Plasmodium* and *Cryptosporidium* genetic divergence demonstrates problems encountered in comparing nucleotide base differences or genetic distances to delineate species. Because the rates of nucleotide substitutions vary among different genes and distantly related organisms, it is difficult to observe generalized patterns that can be applied in species delineation across different taxa. In the case of *Plasmodium* species, both inter- and intraspecific divergence is high [54].

The percentage of sequence divergence is a phenetic character that is not necessarily indicative of phylogenetic divergence as rate heterogeneity and incorrect homology interpretations may provide inaccurate phylogenetic hypotheses. Calculated genetic divergence or evolutionary distance does not provide full information content of phylogenetic divergence and is usually calculated based on a single gene locus. Although sequence differences can be useful diagnostic tools, their use in comparing relative divergence across other species or genera in supporting or refuting species validity [54,92] does not provide true phylogenetic information on which taxonomic classifications should be based. Furthermore, from a purely taxonomic standpoint, no established criteria exist to name species on the basis of nucleotide sequence data for protozoans. For these reasons, divergent sequences or genetic distances between *Cryptosporidium* species must be interpreted with caution in delineating distinct lineages or species.

In another phylogenetic analysis based on SSU rRNA sequences, Xiao *et al* [104,105] determined that the genus *Cryptosporidium* consists of at least four species including *C. parvum*, *C. muris*, *C. baileyi* and *C. serpentis*. The genus was divided into two groups, one containing several *C. parvum* isolates and *C. wrairi* (considered a guinea pig isolate of *C. parvum* [105]) as sister groups and a separate branch with *C. baileyi*, and

the second containing *C. muris* and *C. serpentis* as sister groups. This study supported the distinct nature of the four species listed above. A similar analysis including a sequence for *C. felis* [55] indicated that this species is distinct from *C. parvum*.

Support for additional species of *Cryptosporidium* from SSU rRNA sequences has been proposed in isolates from cats, pigs, and dogs. In a 298-bp fragment of the SSU rRNA gene, Sargent *et al* [73] demonstrated 8.1% divergence between sequences from cat isolates and those from calf and human origins, suggesting that there is a form of *Cryptosporidium* in cats different from those strains infective to humans. A similar study [50] also supported a distinct form of *Cryptosporidium* in cats based on the same 298-bp fragment. Both of these analyses corroborate the findings from analysis of the full SSU rRNA sequence of *C. felis* [55].

The same 298-bp region of the SSU rRNA gene was amplified from DNA of oocysts derived from pigs [52]. A distance-based phylogenetic analysis demonstrated that 8 of 12 samples carried a porcine genotype. The analysis showed three different groups of *Cryptosporidium* including human, bovine, and porcine groups. These studies, as well as those reporting the genotypes of other species such as *C. felis*, provide important preliminary data that indicate genetic heterogeneity among isolates from different host groups. Their characterization will be important for distinguishing different species whose oocysts may not be infective to humans. However, in order to be more effective, the information obtained from different species and isolates will have to be obtained from reference strains that have been passaged through specific hosts and for which DNA can be made available for subsequent analyses.

A recent survey of *Cryptosporidium* oocysts isolated from dogs indicated sequences that differ from several *C. parvum* isolates at two loci, the SSU rRNA gene and the heat shock protein (HSP70) gene [57]. The oocysts were obtained from dogs from Australia and the United States, and both formed a distinct cluster using neighbor-joining and parsimony phylogenetic analyses. From these analyses as well as comparison of genetic similarity (in percentages) among other *Cryptosporidium* species, support for a distinct dog genotype was proposed [57].

Genetic differences between *C. muris* and *C. andersoni* isolates have also been reported. Bovine and camel isolates (isolate A) differed from isolate B identified from mice, hamsters, rock hyrax, and camels [58]. The two morphologically similar species were distinguished on the basis of nucleotide differences in the SSU rRNA gene sequences as well as the ITS1 region and in the HSP70 gene. Originally, both isolates were thought to be *C. muris*, but isolate A has now been recognized as *C. andersoni* [42].

Recent molecular data have been analyzed for *C. meleagridis*. In a multilocus PCR analysis, Champliaud *et al* [23] compared fragments and restriction enzyme patterns in various *C. parvum*, *C. baileyi*, *C. muris* and *C. meleagridis* isolates. The *C. meleagridis* isolate had been obtained from a quail with diarrhea and was subsequently passaged in chickens. Using eight previously published primer pairs from various parts of the *Cryptosporidium* genome, they were unable to differentiate *C. parvum* from *C. meleagridis*. More recently, a phylogenetic analysis of SSU rRNA gene sequences showed that *C. meleagridis* was in a clade containing various *C. parvum* isolates [106]. Here, the *C. meleagridis* isolate was originally isolated from a turkey and cycled in this host. Its close molecular similarity to *C. parvum* did not support a distinct species status [106]. These molecular data reflect the lack of suitable phenotypic criteria to distinguish *C. parvum* from *C. meleagridis*, and the validity of *C. meleagridis* is questionable [82].

Taxonomic descriptions and molecular phylogenetic analyses of these isolates or species should involve data generated from pure experimental infections as oocysts collected from fecal samples may contain several isolates or species of *Cryptosporidium*. Ultimately, the generation of complete SSU rRNA gene sequences as well as other sequences will aid in distinguishing mixed infections, and the isolation and cycling of reference isolates from different hosts will provide a repeatable basis of comparison from different hosts.

In addition to SSU rRNA gene sequences, there is a need for multilocus analyses and other nucleotide sequences may prove useful in resolving the phylogeny of *Cryptosporidium*. HSP70 sequences have recently been obtained for several *Cryptosporidium* species. Phylogenetic analysis of these sequences using the neighbour-joining method and *Plasmodium* sequences to root the trees provided similar hypotheses as for the SSU rRNA gene sequences, namely the presence of a *C. muris*–*C. serpentis* clade and a *C. parvum* clade containing *C. felis*, *C. wrairi*, and *C. meleagridis* sequences as well as those of several *C. pravum* genotypes [85]. The corroboration of current patterns with other sequences could provide a more robust hypothesis explaining the evolution of *Cryptosporidium* species.

Inter- and intraspecific genetic heterogeneity in *Cryptosporidium* based on protein analysis

In addition to DNA sequence information, features of proteins and antigens have been studied for differences among *Cryptosporidium* species and isolates. Isoenzyme typing is a process by which soluble cell extracts are electrophoretically separated on a starch gel by molecular charge. Variations in charge reflected on migration patterns are used to distinguish divergent isoenzymes. In *Cryptosporidium* species, comparative isoenzyme analysis has been performed for glucose phosphate isomerase (GPI), phosphoglucomutase (PGM), lactate dehydrogenase (LDH) and hexokinase (HK) [5,6,63]. Isoenzyme analysis of PGM and GPI, which had much higher activities than other enzymes, allowed differentiation between strains of *C. parvum*, *C. muris* and *C. baileyi*, as all species produced distinct electrophoretic banding patterns [63]. Human and animal isolates of *C. parvum* also produced different banding patterns of PGM and HK [5], suggesting that this species may possess two forms of these enzymes.

Differences in surface antigenic properties have been found between human and animal isolates of *C. parvum* sporozoites. Through western blotting utilizing two monoclonal antibodies developed against *C. parvum*, a 47-kDa major immunodominant sporozoite surface antigen(s) of the human isolate was detected by both antibodies in 9 of 11 isolates from humans in the United Kingdom and Portugal; the monoclonal antibodies recognized one British isolate having an antigen size of 51 kDa, and a Turkish isolate having an antigen size of 45.5 kDa [60]. In contrast, eight bovine and ovine isolates had an antigen size of 48 kDa. These results indicate that the antigenic composition of the sporozoites may differ by parasite geographic distribution.

Genetic heterogeneity in *C. parvum* based on DNA sequences

The sources of oocysts found in water supplies must be identified in attempts to prevent outbreaks of cryptosporidiosis. Potential

Table 2 Studies that have characterized human and animal isolates of *C. parvum* by protein analyses, RAPD, AFLP or PCR-RFLP techniques

Gene/protein	Method	Results	Reference
monoclonal antibodies	reaction of MAb with antigens from various isolates	47-kDa major immunodominant sporozoite surface antigen in humans, 48 kDa in animal isolates	[60]
malate dehydrogenase, carboxylesterase, lactate dehydrogenase, glucose phosphate isomerase, phosphoglucomutase	isoenzyme typing	PGM and GPI zymograms distinguish between *C. parvum, C. muris*, and *C. baileyi*	[63]
glucose phosphate isomerase, lactate dehydrogenase, phosphoglucomutase, hexokinase	isoenzyme typing	distinct human and calf isolates of *C. parvum* shown with HK and PGM	[5]
N.A.	RAPD	distinguished human and animal isolates	[6]
N.A.	RAPD	grouped *C. serpentis*, human isolates, animal isolates	[47]
N.A.	RAPD	four different groups of bovine isolates identified	[74]
N.A.	AFLP	distinguished a human *C. parvum* isolate from various animal isolates	[12]
SSU rRNA gene fragment	PCR-RFLP cut with *Mae*I	*C. parvum* differentiated from *C. baileyi* and *C. muris*	[4]
SSU rRNA gene	PCR-RFLP cut with *Dra*I and *Vsp*I	distinct RFLP profiles for *C. parvum*, *C.muris*, and *C. baileyi*	[38]
C. parvum repetitive DNA sequence	PCR-RFLP cut with 7 restriction enzymes	distinct calf and human isolates	[13]
2.8-kb threonine-rich open reading frame	PCR-RFLP cut with *Rsa*I	distinct calf and human isolates	[20]
ribonucleotide reductase (RNR) R1 subunit	PCR-RFLP cut with *Tsp*509I	distinct calf and human isolates	[99]
ITS1, 5.8S, and ITS2 rDNA	PCR-RFLP cut with *Dra*I	human, cattle, pig, cat, koala, mouse genotypes identified	[53]
COWP, ITS1, TRAP-C1, RNR, polythreonine [poly(T)]	PCR-RFLP	human–animal isolates, human-only isolates identified; 1st nonhuman H isolate identified	[80]
dihydrofolate reductase (DHFR) gene	PCR-RFLP	differentiated human and animal isolates of *C. parvum*	[29]
SSU rRNA gene	PCR-RFLP	differentiated *Cryptosporidium* from other protozoa, identified distinct *Cryptosporidium* spp.	[105]
SSU rRNA gene, others	PCR-RFLP	genotyping of *Cryptosporidium* species and human and bovine *C. parvum* isolates	[84]
COWP, TRAP-C1, SSU rRNA genes	PCR-RFLP	identified two *C. parvum* genotypes; each segregated by COWP and TRAP-C1 analysis	[46]

sources of oocysts of *C. parvum* are from human fecal wastes resulting from either direct contamination or from failure of water treatment systems, and from the feces of domesticated animals, primarily from the livestock industry [27,65,72,97,98]. Development of detection methods for distinguishing oocysts of a human origin from those of bovine hosts has led to discovery of genetic differences in oocysts from both sources (Tables 2 and 3). The approaches listed in Table 2 indicate a gradual progression from protein-based methods to the use of DNA sequences that have distinguished between isolates (Table 3).

Randomly amplified polymorphic DNA analysis (RAPD)

A useful method for surveying overall genetic differences among populations is randomly amplified polymorphic DNA (RAPD) analysis. In this method, genomic DNA is amplified with single, short oligonucleotides. The resulting banding profiles of amplified products can demonstrate distinct patterns in different populations of organisms. A recent RAPD analysis of *Cryptosporidium* DNA obtained from oocysts of human, bovine, and snake origins using several primers revealed a pattern of three distinct groups including *C. serpentis* isolates in one group and two groups of *C. parvum*, from humans in one group and from domestic animals in another [47].

Using an arbitrary primer originally developed to study the multidrug resistance gene in the malaria-causing haemosporinid *Plasmodium falciparum*, Awad-El-Kariem *et al* [6] were able to distinguish between human and animal isolates of *C. parvum*. This 21-base primer produced a four-band RAPD profile for the animal isolates, and a single-band profile for the human isolate. Of the 17 animal isolates (11 bovine and 6 ovine) tested, all showed the same four-band pattern. A single band profile was observed for 9 of the 15 human isolates, with the remaining 6 isolates showing a pattern identical to the animal four-band pattern [6]. Interestingly, patterns of isozyme electrophoresis for PGM corresponded to the RAPD profiles generated for the isolates.

The RAPD method was also used to differentiate four different groups of bovine strains in Minnesota and North Dakota [74]. Here, the use of various RAPD primers suggested the presence of several different *C. parvum* strains within a small geographic area.

The RAPD method may have suitable application in distinguishing between isolates. The differences observed between human and bovine isolates suggest that this method may be used in differentiating human-derived from bovine-derived oocysts. However, it will be necessary to use these primers with pure isolates

Table 3 Genetic analyses that have characterized human and animal isolates of *C. parvum* by direct DNA sequence analyses

Gene	Method	Results	Reference
SSU rRNA and ITS1	RAPD, sequence analysis	human isolates and isolates from various other hosts distinguished	[19]
SSU rRNA 298-bp region	sequence analysis	distinct differences between human, calf, and koala isolates	[49]
SSU rRNA 298-bp region and acetyl-CoA synthetase gene 390-bp region	sequence analysis	"calf" genotype common to cattle, sheep, goats; "human" isolate also identified; also, mouse, pig, snake	[51]
SSU rRNA partial sequence, ITS1, and HSP70	sequence analysis	*C. andersoni*, *C. muris* distinct	[58]
SSU rRNA 298-bp region, HSP70, and acetyl coenzyme A synthetase	sequence analysis	identification of H, C *C. parvum*, *C. meleagridis*, and *C. felis* in humans	[56]
SSU rRNA partial sequence, HSP70	sequence analysis	"dog" genotype of *C. parvum* identified	[57]
β-tubulin intron	sequence analysis	distinct C and H isolates, other alleles detected	[100]
β-tubulin gene fragment	sequence analysis, PCR-RFLP	distinct human isolate, distinct human and animal isolate identified	[16]
β-tubulin gene intron	sequence analysis	distinguished human *C. parvum* isolates	[71]
Cryptosporidium COWP partial gene sequence (550 bp)	sequencing, PCR-RFLP	distinguished *C. parvum* from *C. wrairi*, identified two groups of *C. parvum* differentially associated with animal and human infections	[78]
Thrombospondin-related adhesive protein of *Cryptosporidium*-1 (TRAP-C1)	sequencing and PCR-RFLP	animal–human and human isolates of *C. parvum* distinguished	[80]
Thrombospondin-related adhesive protein (TRAP-C2) of *C. parvum*, 369-bp fragment	sequencing	animal–human and human-only isolates identified	[67]
Thrombospondin-related adhesive protein (TRAP-C2) of *C. parvum*, 369-bp fragment	sequencing and PCR-RFLP	human and bovine genotypes identified	[83]

to avoid the possibility of contamination with other isolates. The patterns derived from pure isolates can then be compared to environmental samples in subsequent screening tests. The RAPD method has the drawback that it may be difficult to obtain consistent results as variation in reaction conditions or template DNA may yield different patterns.

In a different approach, RAPD has been used to generate diagnostic primers suitable for specific detection of *C. parvum* [48]. In this method, RAPD was first used to produce banding patterns from various *C. parvum* isolates. Through Southern hybridization, a band that bound *C. parvum* DNA specifically was isolated. The sequence of this band was then used to design nested primers for the PCR detection of *C. parvum* DNA. A 680-bp fragment specific for various *C. parvum* isolates was produced by this method.

Amplified fragment length polymorphism (AFLP) analysis

Recently, amplified fragment length polymorphism (AFLP) has been introduced as a technique for DNA fingerprinting [96]. Here, restriction fragments from a digest of total genomic DNA are amplified by PCR. Polymorphisms are identified by the presence or absence of DNA fragments, following restriction and amplification of genomic DNA [11]. The method was recently used to investigate genetic variability of 10 *C. parvum* isolates of human and animal origin [12]. A set of nine primer pairs was found to provide consistent patterns for each isolate tested. Of the ten isolates, all isolates of animal origin showed a distinct pattern of fragment sizes and a different pattern was observed in an isolate of *C. parvum* from humans. The AFLP method is thus useful in distinguishing isolates of *C. parvum* from human and bovine origins. One drawback with AFLP is that a large amount of DNA is required.

PCR-RFLP analysis

Another method of detecting genetic differences in populations without sequencing PCR products involves amplifying a sequence and cutting the product with restriction enzymes to analyze restriction fragment length polymorphism (PCR-RFLP). The resulting banding patterns from different groups are compared. In an application of this method, Awad-El-Kariem *et al* [4] amplified a small portion of the SSU rRNA gene from three *Cryptosporidium* species by PCR and restricted the products with *Mae*I. The resulting banding patterns of *C. parvum* differed from those of *C. baileyi* and *C. muris*. The SSU rRNA gene was also used in a PCR-RFLP analysis by Leng *et al* [38] who were able to differentiate *C. parvum*, *C. muris* and *C. baileyi* by comparing RFLP profiles of the amplified SSU rRNA gene digested with *Dra*I and *Vsp*I.

Using a similar method, Bonnin *et al* [13] amplified a *Cryptosporidium*-specific fragment by PCR and subjected it to restriction digestion with seven enzymes. The PCR product was of a repeated DNA sequence initially screened from a genomic DNA library. Ten out of ten calf isolates had a similar profile. Seven of 13 human isolates showed the calf profile whereas six showed a different profile, indicating that a strain different from the calf isolates may occur in humans.

Recently, the ITS1, 5.8S and ITS2 regions were analyzed in *Cryptosporidium* species from different hosts by sequence and PCR-RFLP analyses [53]. The human isolates from different geographic locations were virtually identical as were calf isolates from different locations. However, genetic differences between other *Cryptosporidium* isolates (i.e., from alpaca, sheep, mouse, deer, pig, and cat hosts) were more extensive in these regions of the rDNA [53]. RFLP analysis (using *Dra*I) identified six distinct genotypes designated human, cattle, pig, cat, koala, and mouse [53]. The extensive genetic differences in the ITS region among various *Cryptosporidium* species suggest that this region of the

genome may not provide suitable phylogenetic characters to resolve the relationships of distantly related isolates.

A comparison of various PCR-RFLP protocols for genotyping *Cryptosporidium* species [84] revealed that two *C. parvum* genotypes could be differentiated using ribosomal RNA primers that had previously been described [19,49]. Similar results were found for various other protocols, and the most sensitive protocols evaluated were nested PCR-RFLP protocols described by Gibbons *et al* [29] and Xiao *et al* [105]. By contrast, other previously published protocols were less reliable in distinguishing between *C. parvum* genotypes and, in some cases, were not *Cryptosporidium*-specific [84]. Development of a sensitive PCR-RFLP protocol may provide a reliable method for identifying genotypes. At present, the heterogeneity of *C. parvum* needs to be further characterized by additional methods such as direct sequencing of various loci before the utility of PCR-RFLP can be evaluated. The latter method must also be applied to a wider range of *Cryptosporidium* species to further assess its diagnostic potential. Although the results presented so far using several loci are promising, the possibility of cross contamination with more than one *Cryptosporidium* species or isolate in field samples continues to be as problematic as the other molecular diagnostic methods currently available.

Interestingly, there is a possibility of genotypic changes occurring when *C. parvum* is transmitted from bovine to human hosts. Carraway *et al* [20] assessed RFLP within a 2.8-kb threonine-rich open reading frame from *C. parvum* using *Rsa*I. They found that all calf isolates examined shared the same profiles whereas human isolates included a unique profile as well as the calf profile. Two of the isolates used, GCH4 and GCH5 were considered to be human isolates obtained from workers who may have become infected with GCH1, a calf isolate with which they had been working. The ribosomal and *Rsa*I fingerprints indicated a change in genotype between GCH1 and GCH4–GCH5 [20]. Samples of GCH4 and GCH 5 were found to have both *Rsa*I-positive and *Rsa*I-negative restriction sites, suggesting genetic heterogeneity.

Intraspecific variation in *C. parvum*-DNA sequences

Attempts are being made to corroborate the differences in human and bovine *C. parvum* isolates detected by RAPD and PCR-RFLP techniques by direct DNA sequence comparison of both groups, and evidence of two distinct *C. parvum* human and animal genotypes is accumulating (Table 3). By comparing SSU rRNA and ITS-1 gene sequences in known *C. parvum* isolates, Carraway *et al* [19] described base differences in several groups. PCR primers were designed to allow differentiation of TGA polymorphism at positions 645–647 of an SSU rRNA gene fragment. Sequenced products indicated a TGA deletion in several isolates, and there was extensive polymorphism of ITS-1 sequences. However, on the basis of the differences in both SSU rRNA gene sequences as well as ITS-1, the five isolates of *C. parvum* examined could be segregated into two groups consisting of human isolates and isolates propagated in laboratory animals [19].

Partial SSU rRNA gene sequences have been used [49] to demonstrate that short regions in a 298-bp region differed by several bases between human and animal isolates. An isolate from a koala was also found to vary from both human and other animal isolates. The same region of the gene was amplified in *Cryptosporidium* species from other hosts including snakes, alpacas, sheep, goats, mice, and humans, and a distinct "calf" genotype was identified using the SSU rRNA fragment as well as an acetyl-CoA synthetase gene sequence [51]. Distinct mouse, pig, and snake genotypes were also identified.

Recently, a detailed study of the ribosomal RNA genes of *C. parvum* was conducted [37]. It was found that there are five copies of the total rDNA unit per haploid genome in the KSU-1 isolate. Two structurally distinct forms of the rDNA unit, designated types A and B, were discovered. There were several nucleotide differences between both types [37], particularly in the ITS-1 region. These findings indicate that various differences found in the rRNA gene sequences of *C. parvum* isolates may be sequences of either of the two rDNA types. In future analyses using this gene, attempts should be made to determine the rDNA type being sequenced.

Two types of SSU rRNA transcripts have been identified in *C. parvum* in both oocysts and intracellular stages. Using RT-PCR and denaturing gradient gel electrophoresis, Widmer *et al* [101] identified two types of transcripts from bovine calf-propagated *C. parvum* isolates. The presence of several different types of rRNA genes in *C. parvum* is similar to the multiple genes that have been described in the malaria-causing *Plasmodium* species [45,101].

The use of SSU rRNA gene sequences to distinguish *Cryptosporidium* isolates and species is of value in comparing distantly related species. The main species groups described by Xiao *et al* [104,105] are well resolved phylogenetically based on these sequences. However, relationships among the more closely related species as well as isolates within *C. parvum* are not well resolved using SSU rRNA sequence information. The lack of suitable phylogenetic information content in the SSU rRNA gene sequence among closely related taxa, coupled with the presence of more than one form of the gene in individual organisms, indicate that other loci must be evaluated for more resolved phylogenetic analyses and genotyping methods.

Other gene sequences have been investigated to search for differences in *C. parvum* isolates, and a consistent pattern is emerging depicting a human genotype and a calf genotype, designated as the H and C types, respectively [99]. The H and C types have been distinguished by differences in the β-tubulin intron and isolates with several different β-tubulin alleles have been found [100]. In another study, a fragment of the β-tubulin intron and part of the coding region was amplified and sequenced, and differences at 12 bases corresponding to the human and animal genotypes were identified [16]. The two alleles at the β-tubulin locus could be identified by digestion of the PCR products with *Dde*I or *Hae*III [16]. Polymorphism in an intron of the β-tubulin gene was also used to distinguish between isolates of *C. parvum* from humans and isolates from both animals and humans with a sequence divergence of 1.8% [71]. These studies from different investigators indicate the utility of this locus as an indicator of genotype difference in *C. parvum* isolates, although geographic differences among the same isolates could not be found [71].

The *Cryptosporidium* oocyst wall protein (COWP) gene was recently sequenced and analyzed in *C. parvum* [77], and its utility in distinguishing *Cryptosporidium* species was indicated by Spano *et al* [78], who compared a part of the COWP gene sequence in several isolates of *C. parvum* and in *C. wrairi*. The partial sequences of approximately 550 bp were 98–99% identical in *C. parvum* and *C. wrairi*. A PCR-RFLP analysis of the sequence identified an animal isolate in animals and humans as well as an

isolate exclusively in humans [78]. Using COWP polymorphism, two *C. parvum* genotypes were identified by McLaughlin *et al* [46], who found that the same *C. parvum* genotypes distinguished by COWP polymorphisms also segregated with those genotypes identified by thrombospondin-related adhesive protein C1 (TRAP-C1) polymorphisms.

The TRAP-C1 gene also appears to be a promising marker that can be used to distinguish between human and animal isolates. TRAP-C1 proteins are localised in the apical end of sporozoites and are structurally related to micronemal proteins of other apicomplexans, i.e., *Toxoplasma* and *Eimeria* [79]. Spano *et al* [80] used restriction enzyme digestion of TRAP-C1 PCR amplicons to demonstrate that two animal isolates and one isolate from humans had a similar pattern that differed from a pattern found in three other isolates from humans. Sequencing cloned PCR products from a cervid isolate and a human isolate showed distinct nucleotide differences in two regions of the gene [80]. The animal and human profiles of the six isolates tested in this study corresponded to those detected by COWP gene analysis [78].

A similar gene, TRAP-C2, has also been used to differentiate between the human and animal isolates [67,83]. This gene, like TRAP-C1, is a thrombospondin-related protein having multiple copies of a thrombospondin-related motif [79]. Thirty-nine isolates of *C. parvum* were analyzed for polymorphism in a 369-bp portion of the TRAP-C2 gene [67]. Alignment of various sequences indicated the presence of two primary genotypes based on nucleotide differences at five different positions in the sequence. One genotype contained human isolates whereas the second contained both human and calf isolates. Experimental infection studies suggested that two distinct populations of *C. parvum* were cycling in humans as genotype 1 isolates from humans did not infect mice or calves [67]. In an extended study using 92 isolates, Sulaiman *et al* [83] confirmed the previous results that two alleles of the TRAP-C2 gene exist, each corresponding to a distinct genotype of *C. parvum* with different transmission cycles in humans. Here, a PCR-RFLP protocol was described whereby bovine and human genotypes could be distinguished.

The identification of H and C *C. parvum* isolates was further tested on 28 isolates using five polymorphic loci [81]. Using COWP, ITS1, TRAP-C1, ribonucleotide reductase (RNR), and polythreonine [poly(T)], distinct H and C genotypes were characterized by PCR and PCR-RFLP profiles. PCR products for each of the loci were cut with various restriction enzymes and distinct electrophoretic patterns were observed for each of the two genotypes [81].

Recently, the H and C genotypes have been further divided into "subgenotypes" using microsatellite markers [17]. *C. parvum* DNA was isolated from 48 human and 46 animal sources. A locus containing a GAG microsatellite was amplified by PCR and sequenced. Within this region, human *C. parvum* subgenotypes designated H1 and H2 could be distinguished by the presence of a $(GAA)_2$ motif in H2. Four subgenotypes of the C isolate, designated C1–C4 were identified from the same amplified sequence [17]. These findings indicate the potential to further resolve closely related genotypes of the two *C. parvum* isolates.

Future perspectives

The interest that *Cryptosporidium* has generated in recent years has resulted in advances in the typing of species and isolates as well as in the systematics of the genus. A number of sequences for different isolates have accumulated in molecular databases. These sequences, as used in studies to differentiate species and isolates of *Cryptosporidium* species, have exposed several difficulties in studying this group at both phenotypic and genotypic levels. Other methods for identifying genotypes, such as AFLP analyses [11,12] show promise in helping to identify *Cryptosporidium* species and isolates.

In addition to gene loci currently used to characterize species and isolates, other genetic data must be assessed to study speciation in *Cryptosporidium*. Gene mapping of the entire *C. parvum* genome will provide valuable new data for this purpose. A "HAPPY" map of *C. parvum* has been constructed [69]. The method uses an *in vitro* linkage technique that screens approximately haploid amounts of DNA by PCR. The map covers all eight chromosomes [69]. By random analysis of the *C. parvum* genome, Liu *et al* [43] obtained sequence contigs representing approximately 2.5% of the genome. Comparison of the sequences with previously identified proteins and genes demonstrated several similarities to known genes from other organisms.

There is also currently a *C. parvum* genome sequencing project underway in which greater than 99% of the genome is to be sequenced for two *C. parvum* isolates. This is a collaborative effort from Virginia Commonwealth University, Tufts University School of Medicine, University of Minnesota, Stanford University and the University of California San Francisco. Various clones and Expressed Sequence Tags (ESTs) are currently available in the GenBank database (http://www.ncbi.nlm.nih.gov/dbEST/index.html). This project is aiming to provide the same advantages in sequencing the entire genome of *C. parvum* as have been found from genome sequencing projects for other organisms.

One of the main problems in characterizing genomes of parasitic and other organisms is the lack of reference material for future research. If an isolate of a parasite is collected from field samples, subsequent studies may not be possible if more samples are unavailable. Genotypic characterization of *Cryptosporidium* species should be accompanied with as much additional information as possible. For example, photographic data of oocysts as well as detailed morphometric measurements [7] can provide a basis for identifying future sources of parasites. If possible, oocysts should be cycled through additional hosts before molecular systematic studies are undertaken. This may avoid the possibility of mixed isolates or species being present in a sample. Recently, a genotype 1 isolate of *C. parvum*, the human isolate, has been successfully cycled in piglets [102]. A genomic library was constructed from these parasites. This work will allow a detailed characterization of the human *C. parvum* genotype and has demonstrated successful propagation of the parasites in animals.

The absence of defined and characterized reference strains has made the basis for speciation of *Cryptosporidium* ambiguous. The lack of defined phenotypic and genotypic parameters demonstrates difficulties in comparing species and strains [92]. In addition, because of the variety of hosts and cell types that *Cryptosporidium* can infect, Tzipori and Griffiths [92] felt it to be premature to "firmly divide *Cryptosporidium* into valid species without further studies." However, the data we have reviewed here indicate that there are observable differences between various species both at phenotypic and genotypic levels. There is also an increasing body of evidence demonstrating two isolates of *C. parvum* in human and bovine hosts. The naming and identification of different species has been difficult because traditional species concepts are difficult to

apply to closely related taxa such as *Cryptosporidium* species. Molecular data are becoming increasingly important in distinguishing closely related groups, and such data will have to be incorporated into a species definition that includes a ''diagnosability'' criterion as in the phylogenetic species concept of Nixon and Wheeler [61].

Problems in isolating and propagating isolates of *Cryptosporidium* have been described [98]. Mixed infections will likely be encountered frequently. However, elucidation of marker sequences for certain species and strains will eventually enable researchers to identify mixed infections and the isolates available in a sample. A combination of data on a given isolate with the availability of reference material (e.g., oocysts propagated in laboratory hosts, genomic DNA libraries, and frozen oocysts or DNA for future analyses, photographic voucher material) will eventually determine patterns that will better characterize *Cryptosporidium* species. These patterns are vital in developing accurate detection methods for the parasites and may provide information on the relative pathogenicity of isolates.

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