

Phylogenetic Analysis of *Cryptosporidium* Parasites Based on the Small-Subunit rRNA Gene Locus

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Biological data support the hypothesis that there are multiple species in the genus *Cryptosporidium*, but a recent analysis of the available genetic data suggested that there is insufficient evidence for species differentiation. In order to resolve the controversy in the taxonomy of this parasite genus, we characterized the small-subunit rRNA genes of *Cryptosporidium parvum*, *Cryptosporidium baileyi*, *Cryptosporidium muris*, and *Cryptosporidium serpentis* and performed a phylogenetic analysis of the genus *Cryptosporidium*. Our study revealed that the genus *Cryptosporidium* contains the phylogenetically distinct species *C. parvum*, *C. muris*, *C. baileyi*, and *C. serpentis*, which is consistent with the biological characteristics and host specificity data. The *Cryptosporidium* species formed two clades, with *C. parvum* and *C. baileyi* belonging to one clade and *C. muris* and *C. serpentis* belonging to the other clade. Within *C. parvum*, human genotype isolates and guinea pig isolates (known as *Cryptosporidium wrairi*) each differed from bovine genotype isolates by the nucleotide sequence in four regions. A *C. muris* isolate from cattle was also different from parasites isolated from a rock hyrax and a Bactrian camel. Minor differences were also detected between *C. serpentis* isolates from snakes and lizards. Based on the genetic information, a species- and strain-specific PCR-restriction fragment length polymorphism diagnostic tool was developed.

Cryptosporidiosis is a coccidian infection of humans, domestic animals, and wild vertebrates. In farm animals, *Cryptosporidium* parasites cause disorders of the digestive and respiratory systems, which lead to poor health of infected animals and significant economic losses. In immunocompetent humans, *Cryptosporidium* parasites cause acute infections of the digestive system, but in immunocompromised patients they cause a chronic, life-threatening disease. The general consensus is that one species, *Cryptosporidium parvum* from mammals, is the only *Cryptosporidium* species responsible for infections in humans (11, 24). In contrast, a recent study suggested that all *Cryptosporidium* parasites, including those from lower vertebrates, should be considered hazardous to humans (35).

The contrasting views are largely the result of confusion in the taxonomy of *Cryptosporidium* parasites. Since the discovery of *Cryptosporidium muris* and *C. parvum* in rodents at the turn of the century (31–33), more than 20 *Cryptosporidium* species from various animals have been described (24). Studies conducted in late 1970s and early 1980s, however, indicated that cross-transmission of some isolates among various animal species was possible. Thus, it was suggested that all *Cryptosporidium* parasites belong to the same species, *C. muris* (34). Later, it was shown that various *Cryptosporidium* isolates (at least isolates obtained from members of different classes of vertebrates) exhibit host specificity. Based on these observations, Levine (18, 19) proposed that the parasites from mammals, birds, reptiles, and fish should be classified as *C. muris*, *Cryptosporidium meleagridis*, *Cryptosporidium serpentis*, and

Cryptosporidium nesorum, respectively. Subsequently, it was realized that *C. parvum* from mammals and *Cryptosporidium baileyi* from birds are biologically and morphologically different from *C. muris* and *C. meleagridis* (8, 36). Thus, *C. parvum*, *C. muris*, *C. baileyi*, *C. meleagridis*, *C. serpentis*, and *C. nesorum* have been considered the six valid *Cryptosporidium* species by many researchers (24). More recently, based on host specificity data, Fayer et al. (11) added *Cryptosporidium felis* from cats and *Cryptosporidium wrairi* from guinea pigs to the list of valid species. The taxonomy of the genus *Cryptosporidium* was recently challenged by a recent study which suggested that genetic data do not support the currently proposed species designations (35).

In this study, we compared *Cryptosporidium* isolates from humans and various animals at the 18S small-subunit (SSU) rRNA gene locus. The results of our study revealed that the genus *Cryptosporidium* consists of multiple species, as proposed previously on the basis of biological characteristics and host specificity data. The species analyzed in this study formed two clades; *C. parvum* and *C. baileyi* were most closely related to each other in one clade, and *C. muris* and *C. serpentis* were most closely related to each other in the other clade. The differences in the rRNA gene at the species and strain levels allowed us to develop specific diagnostic procedures for accurate identification of *Cryptosporidium* parasites in clinical and environmental samples.

MATERIALS AND METHODS

***Cryptosporidium* isolates and oocyst and DNA isolation.** The isolates used in this study were obtained from humans, cattle, snakes, lizards, a guinea pig, a camel, a hyrax, and a chicken (Table 1). Oocysts were purified from fecal samples by a combination of discontinuous density sucrose gradient centrifugation and isopycnic Percoll centrifugation or cesium chloride gradient centrifugation. After treatment in a 5.25% sodium hypochlorite solution (100% commercial bleach) at

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TABLE 1. Isolates of *Cryptosporidium* parasites used in this study^a

Isolate	Species	Host	Location	Length of SSU ribosomal DNA (bp)	A + T content (%)
HFL2	<i>C. parvum</i>	Human	Florida	1,750	60.97
HFL5	<i>C. parvum</i>	Human	Florida	1,745	60.86
HCVN4	<i>C. parvum</i>	Human	Nevada	1,750	61.03
DR1	<i>C. parvum</i>	Deer via calf	Georgia	1,746	60.88
GCH1	<i>C. parvum</i>	Human via calf	Massachusetts	1,746	60.88
BOH6	<i>C. parvum</i>	Calf	Ohio	1,746	60.88
GP1	<i>C. wairi</i>	Guinea pig	Michigan	1,746	60.71
CSP01	<i>C. serpentis</i>	Corn snake	Kansas	1,743	58.52
CSP05	<i>C. serpentis</i>	Amazon tree boas	Washington, D.C.	1,743	58.52
CSP02	<i>C. serpentis</i>	Savannah monitor	Washington, D.C.	1,743	58.29
CSP04	<i>C. serpentis</i>	Savannah monitor	Washington, D.C.	1,743	58.52
IDVS-811	<i>C. muris</i>	Cattle	Idaho	1,743	57.74
IDRH-13	<i>C. muris</i>	Rock hyrax via mouse	Washington, D.C.	1,746	57.85
CMU03	<i>C. muris</i>	Bactrian camel via mouse	Washington, D.C.	1,746	57.85
CBA01	<i>C. baileyi</i>	Chicken	Alabama	1,733	58.80

^a Partial sequences (length, about 790 bp) were also obtained from four additional human genotype isolates, 18 bovine genotype isolates, and one *C. baileyi* isolate.

4°C for 10 min, oocysts were washed five times in sterile water. DNA was extracted from purified oocysts by a previously described technique (14).

PCR, cloning, and DNA sequencing. The full-length SSU rRNA gene was amplified from each sample by conventional PCR by using forward primer 5'-AACCTGGTTGATCCTGCCAGTAGTC-3' and reverse primer 5'-TGATCCTTCTGCAGGTTACCTACG-3'. Each PCR consisted of 35 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 60 s; an initial denaturation step consisting of incubation at 94°C for 5 min and a final extension step consisting of incubation at 72°C for 10 min were also included. After PCR amplification, the PCR fragment was cloned into the CloneAmp pAMP1 system (Gibco BRL, Frederick, Md.). At least two positive clones from each sample were sequenced by using a model ABI377 autosequencer (Perkin Elmer, Foster City, Calif.).

Sequence analyses. The DNA sequences obtained from two different clones of each isolate were compared with each other first. Any discrepancy between the two sequences was resolved by the sequence obtained from a third clone. The sequences were aligned by using the Clustal W program (30) with manual adjustment (a data matrix used in the analysis is available upon request). We performed two phylogenetic analyses. First, a neighbor-joining (NJ) tree (27) was constructed for all species belonging to the Apicomplexa in the study. The tree was rooted by using sequences from dinoflagellates (*Perkinsus*, *Gymnodinium*, and *Gyrodinium* spp.), which are closely related to apicomplexans (10, 17). In the second analysis, we restricted the analysis to the *Cryptosporidium* species. The reliability of the NJ tree was assessed by the bootstrap method (12) with 1,000 replications. We used 95% as the statistically significant value (9); however, values greater than 70% are reported below since bootstrap values may be conservative estimates of the reliability of clades (13). NJ and bootstrap analyses were performed by using the program Treecon W (37). The relative distances among different *Cryptosporidium* spp. were calculated by using the Kimura two-parameter method and the Wisconsin Package, version 9.0 from the Genetics Computer Group. SSU rRNA sequences of *Babesia divergens* (accession no. U16370), *Theileria parva* (L02366), *Cytauxzoon felis* (L19080), *Eimeria tenella* (AF026388), *Eimeria praecox* (U67120), *Cyclospora* sp. (U40261), *Isospora suis* (U97523), *Toxoplasma gondii* (U03070), *Neospora caninum* (U03069), *Sarcocystis tenella* (L24383), *Frenkelia microti* (AF009244), *Gymnodinium simplex* (U41086), *Gymnodinium* sp. (AF022196), *Gyrodinium impudicum* (AL022197), and *Perkinsus marinus* (X75762) were obtained from the GenBank database.

PCR-RFLP analysis. To develop a PCR-restriction fragment length polymorphism (RFLP) technique for species- and strain-specific diagnosis of *Cryptosporidium* parasites, sequences unique to all *Cryptosporidium* species were identified from the multiple alignment and used as primers in a nested PCR protocol. For the primary PCR step, a PCR product that was about 1,325 bp long was amplified by using primers 5'-TTCTAGAGCTAATACATGCG-3' and 5'-CCCTAATCC TTCGAAACAGGA-3'. Each PCR mixture (total volume, 100 µl) contained 10 µl of Perkin-Elmer 10× PCR buffer, 6 mM MgCl₂, each deoxynucleoside triphosphate at a concentration of 200 µM, each primer at a concentration of 200 nM, 2.5 U of *Taq* polymerase, and 0.25 to 2 µl of DNA template. A total of 35 cycles, each consisting of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, were performed; an initial hot start at 94°C for 3 min and a final extension step at 72°C for 7 min were also included. For the secondary PCR step, a PCR product that was 819 to 825 bp long (depending on the species) was amplified by using 2 µl of the primary PCR product and primers 5'-GGAAGGGTGTATTATTAGAT AAAG-3' and 5'-AAGGAGTAAGGAACAACCTCCA-3'. The PCR mixture and cycling conditions were identical to the conditions used for the primary PCR step, except that 3 mM MgCl₂ was used in the PCR mixture.

For restriction fragment analysis, 20 µl of the secondary PCR products was digested in a 50-µl reaction mixture containing 20 U of *SspI* (New England BioLabs, Beverly, Mass.) (for species diagnosis) or 20 U of *VspI* (Gibco BRL, Grand Island, N.Y.) (for genotyping of *C. parvum*) and 5 µl of the appropriate restriction buffer at 37°C for 1 h, under conditions recommended by the supplier. The digested products were fractionated on a 2.0% agarose gel and visualized by ethidium bromide staining.

Nucleotide sequence accession numbers. The nucleotide sequences of the SSU rRNA genes of *C. parvum*, *C. wairi*, *C. muris*, *C. baileyi*, and *C. serpentis* have been deposited in the GenBank database under accession no. AF093489 to AF093502 and AF115377.

RESULTS

Complete sequences of the SSU rRNA gene were obtained for six *C. parvum* isolates, one *C. wairi* isolate, one *C. baileyi* isolate, three *C. muris* isolates, and four *C. serpentis* isolates. The length of the gene varied from 1,733 to 1,750 bp depending on the species and isolate; the gene of the *C. parvum* human genotype (HFL2 and HCVN4) was the longest, and the *C. baileyi* gene was the shortest (Table 1). The rRNA genes of the *Cryptosporidium* parasites were AT rich, with A+T contents of 57.74 to 61.03%. Within each species, however, the A+T contents of different isolates were quite similar. All bovine *C. parvum* SSU rRNA sequences were from the type A unit (15).

To establish the relationship between *Cryptosporidium* spp. and other apicomplexans, the SSU rRNA gene sequences of *Cryptosporidium* spp. were aligned with the previously published sequences of *B. divergens* (accession no. U16370), *T. parva* (L02366), *C. felis* (L19080), *E. tenella* (AF026388), *E. praecox* (U67120), *Cyclospora* sp. (U40261), *I. suis* (U97523), *T. gondii* (U03070), *N. caninum* (U03069), *S. tenella* (L24383), *F. microti* (AF009244), *Gymnodinium* sp. (AF022196), *G. simplex* (U41086), *G. impudicum* (AL022197), and *P. marinus* (X75762) obtained from GenBank. An NJ tree was constructed by using the dinoflagellate parasites as an outgroup (Fig. 1A). The five *Cryptosporidium* species formed a monophyletic group in the tree, with full statistical reliability (bootstrap value, 100%). Other apicomplexans formed a separate clade, with a bootstrap value of 77%. Within the latter clade the non-*Cryptosporidium* members of the suborder Eimeriorina (members of the genera *Eimeria*, *Cyclospora*, *Sarcocystis*, *Isospora*, *Toxoplasma*, *Frenkelia*, and *Neospora*) formed a monophyletic group with full statistical reliability. Among the non-*Cryptosporidium* coccidians, the parasites once historically

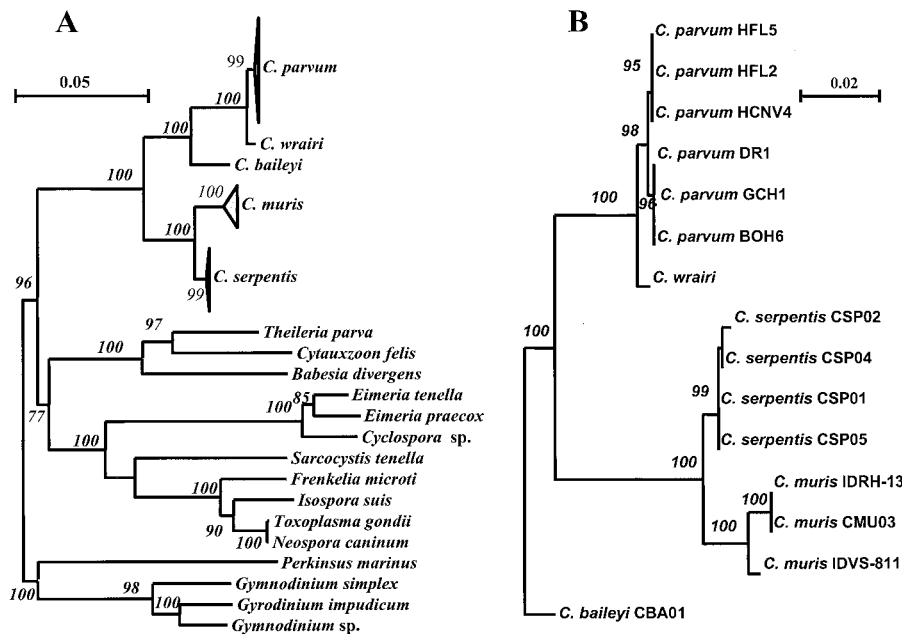


FIG. 1. Phylogenetic relationships of *Cryptosporidium* parasites to other apicomplexan parasites (A) and to each other (B).

placed in the genus *Isospora*, such as *Isospora* spp., *Sarcocystis* spp., *Toxoplasma* spp., *Frenkelia* spp., and *Neospora* spp. (17), formed a monophyletic group distinct from the classical coccidian genus *Eimeria* and the recently identified genus *Cyclospora*.

The five *Cryptosporidium* species examined in this study formed two clades with full statistical reliability (Fig. 1A). One clade contained *C. parvum*, *C. wrairi*, and *C. baileyi*, and *C. parvum* and *C. wrairi* grouped together in this clade. The separation of *C. baileyi* from *C. parvum* and *C. wrairi* and the separation of *C. parvum* from *C. wrairi* were statistically reliable (bootstrap values, 99 to 100%). The other clade consisted of *C. muris* and *C. serpentis*, which were separated from each

other reliably (bootstrap values, 99 to 100%). Most of the interspecies differences in primary sequences occurred in the first half of the SSU rRNA gene.

Intraspecies variations were also found in *C. parvum*, *C. muris*, and *C. serpentis*, and the most noteworthy observations were made with *C. parvum* (Fig. 1B and Table 2). There were two genotypes of *C. parvum*, which differed from each other in four areas of the SSU rRNA gene (data not shown). The human genotype included the three isolates from humans, whereas the bovine genotype included the three bovine isolates that originated from cattle, deer, and human sources (Fig. 1B). The genotype of the closely related *Cryptosporidium* isolate from guinea pigs (designated *C. wrairi*) was also different from

TABLE 2. Evolutionary distances among *Cryptosporidium* spp. and other organisms^a

Organism	No. of nucleotide differences/100 bases														
	<i>C. parvum</i> HFL2	<i>C. parvum</i> HFL5	<i>C. parvum</i> HCNV4	<i>C. parvum</i> DR1	<i>C. parvum</i> GCH1	<i>C. wrairi</i>	<i>C. baileyi</i>	<i>C. serpentis</i> CSP01	<i>C. serpentis</i> CSP05	<i>C. serpentis</i> CSP04	<i>C. serpentis</i> CSP02	<i>C. muris</i> CMU03	<i>C. muris</i> IDRH-13	<i>C. muris</i> IDVS-811	<i>E. tenella</i>
<i>C. parvum</i> HFL5	0.11														
<i>C. parvum</i> HCNV4	0.11	0.11													
<i>C. parvum</i> DR1	0.23	0.17	0.23												
<i>C. parvum</i> GCH1	0.23	0.17	0.23	0											
<i>C. wrairi</i>	0.63	0.58	0.63	0.46	0.46										
<i>C. baileyi</i>	3.93	3.93	3.93	3.69	3.69	3.69									
<i>C. serpentis</i> CSP01	5.87	5.82	5.87	5.81	5.81	5.63	5.08								
<i>C. serpentis</i> CSP05	5.87	5.82	5.87	5.81	5.81	5.63	5.08	0							
<i>C. serpentis</i> CSP04	5.99	5.94	5.99	5.94	5.94	5.75	5.20	0.11	0.11						
<i>C. serpentis</i> CSP02	6.24	6.13	6.24	6.19	6.19	6.00	5.45	0.34	0.34	0.23					
<i>C. muris</i> CMU03	7.00	6.95	7.00	6.89	6.89	7.01	5.71	1.98	1.98	2.10	2.33				
<i>C. muris</i> IDRH-13	7.00	6.95	7.00	6.89	6.89	7.01	5.71	1.98	1.98	2.10	2.33	0			
<i>C. muris</i> IDVS-811	6.74	6.69	6.75	6.51	6.51	6.63	5.46	1.81	1.81	1.92	2.16	0.87	0.87		
<i>E. tenella</i>	20.14	19.95	20.15	20.26	20.19	20.25	19.90	18.52	18.52	18.67	18.90	19.23	19.23	19.16	
<i>Cyclospora</i> sp.	19.86	19.67	19.87	19.84	19.76	19.91	19.60	18.06	18.06	18.21	18.44	18.77	18.77	18.84	3.24

^a Values were calculated by performing a Kimura two-parameter analysis with the Wisconsin Package, version 9.0, of the Genetics Computer Group.

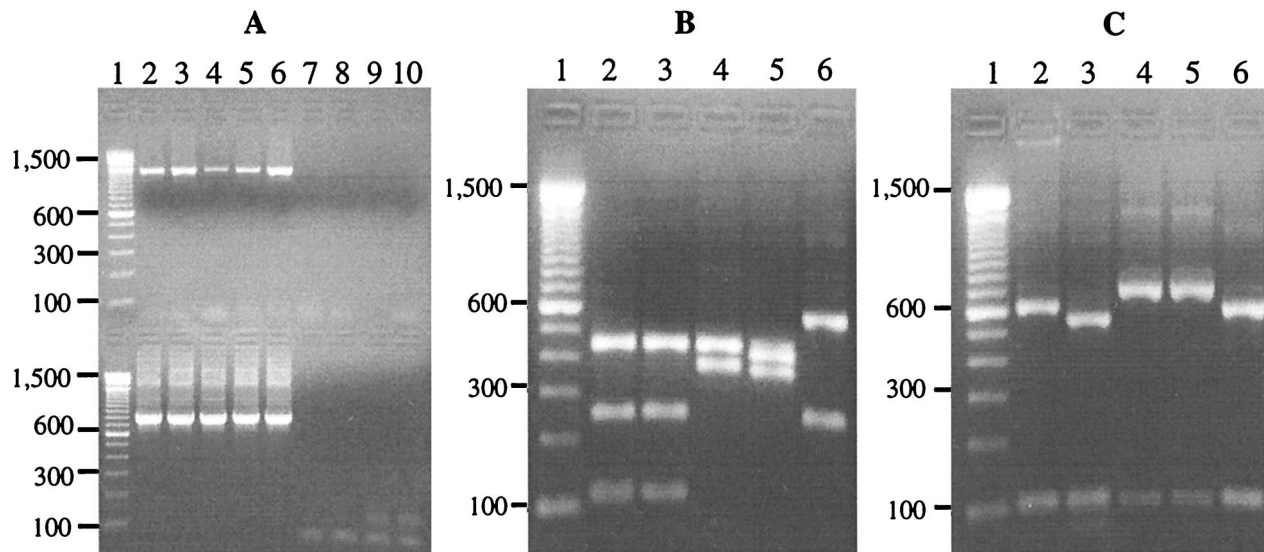


FIG. 2. Molecular diagnosis of *Cryptosporidium* parasites by a nested PCR-RFLP procedure based on SSU rRNA gene sequences. (A) Specific detection of *Cryptosporidium* spp. by nested PCR. Lane 1, molecular weight markers; lane 2, *C. parvum* bovine genotype; lane 3, *C. parvum* human genotype; lane 4, *C. muris*; lane 5, *C. serpentis*; lane 6, *C. baileyi*; lane 7, *Eimeria papillata*; lane 8, *Eimeria nieschulzi*; lane 9, *Giardia duodenalis*; lane 10, negative control. The upper panel contained the primary PCR products, and the lower panel contained the secondary PCR products. (B) Species diagnosis of *Cryptosporidium* parasites by *SspI* digestion of the nested PCR products. For lane contents see above. (C) Differentiation of two genotypes of *C. parvum* by *VspI* digestion of the nested PCR products. For lane contents see above.

the *C. parvum* bovine genotype in four areas. All differences among the three genotypes of *C. parvum* (bovine, human, and guinea pig) occurred in the first half of the SSU rRNA gene. This separation of the three genotypes of *C. parvum* was also evident on the phylogenetic tree (Fig. 1B).

Two genotypes of *C. muris* isolates were also distinguished (Fig. 1B). The SSU rRNA gene sequences of the *C. muris* isolates from a Bactrian camel and a rock hyrax were identical to each other but were different from the SSU rRNA gene sequence of the *C. muris* bovine isolate at 15 nucleotide positions (data not shown). The evolutionary distance (0.87%) between the two *C. muris* genotypes was greater than the evolutionary distances (0.17 to 0.63%) among the three *C. parvum* genotypes (including *C. wairi*) (Table 2). Thus, the two genotypes were separated from each other on the phylogenetic tree with full statistical reliability (Fig. 1B). Unlike the differences in the *C. parvum* genotypes, which occurred in the first half of the SSU rRNA gene, the differences between the two *C. muris* genotypes were spread over the entire gene.

Minor differences were also detected in the SSU rRNA genes of various *C. serpentis* isolates. Although the sequences of the two snake isolates (CSP01 and CSP05) were identical, they differed from the sequences of the two isolates from savanna monitors (lizards) at two to four nucleotide positions (nucleotides 371, 560, 692, and 1688).

Based on the analysis of the SSU rRNA gene sequences, a PCR-RFLP technique was developed for diagnosis of species and strains of *Cryptosporidium* parasites. A 819 to 825-bp product was amplified by a nested PCR from *Cryptosporidium* (*C. parvum*, *C. baileyi*, *C. muris*, and *C. serpentis*) samples used. The *Cryptosporidium* specificity of the PCR was confirmed by the failure to amplify samples of *Eimeria* and *Giardia* parasites (Fig. 2A). This technique was quite sensitive, as demonstrated by amplification of DNA from a single purified oocyst (data not shown). Species diagnoses were made by digesting the secondary PCR product with *SspI* (Fig. 2B). *C. parvum* generated three visible bands of 448, 247, and 106 bp; *C. baileyi*

generated two bands of 572 and 247 bp; *C. muris* generated two bands of 448 and 377 bp; and *C. serpentis* generated two visible bands of 414 and 363 bp. To differentiate human and bovine genotypes of *C. parvum*, the secondary PCR product was digested with *VspI* (Fig. 2C). The *C. parvum* bovine genotype produced two visible bands of 628 and 104 bp, whereas the human genotype produced two visible bands of 556 and 104 bp due to the presence of one additional *VspI* restriction site. In contrast, *C. baileyi* generated a band pattern similar to that of the *C. parvum* bovine genotype after *VspI* digestion, whereas *C. muris* and *C. serpentis* both generated two bands of 724 to 730 and 95 to 100 bp.

DISCUSSION

Phylogenetic studies of the SSU rRNA gene sequences of *Cryptosporidium* parasites have been limited, and the few studies that have been done have contributed to the confusion concerning the systematics of *Cryptosporidium* parasites. An initial SSU rRNA sequence analysis revealed more than 99% identity between *C. parvum* and *C. muris* (4). A recent phylogenetic analysis also failed to separate *C. parvum*, *C. muris*, and *C. baileyi* (35). Sample misidentification and sequence inaccuracy, however, may have affected the outcome of these analyses (40).

Phylogenetic analyses of the SSU rRNA genes of various isolates of *C. parvum*, *C. muris*, *C. baileyi*, and *C. serpentis* in the present study indicated that the *Cryptosporidium* parasites belong to a multispecies complex that can be divided into two groups. The first group includes various isolates of *C. parvum* (including *C. wairi*) and *C. baileyi*, whereas the second group includes all isolates of *C. muris* and *C. serpentis*. The difference between these two groups is greater than the difference between *E. tenella* and *Cyclospora* sp. To minimize the contribution of sequence errors, we used only *Cryptosporidium* SSU rRNA sequences generated in this study in the phylogenetic analysis.

Importantly, the results of the phylogenetic analyses are in agreement with biologic differences between the two groups of *Cryptosporidium* parasites. Biologically, the two *Cryptosporidium* groups have different predilection sites. *C. parvum* and *C. baileyi* primarily infect the small intestine and the respiratory tract, respectively, whereas *C. muris* and *C. serpentis* primarily infect the stomach. Furthermore, some *C. parvum* isolates have been shown to infect chicks when large numbers of oocysts are used for inoculation (20, 34). The results of an analysis of an undefined DNA sequence (39) and an antigen reactivity analysis (23) also suggested that *C. parvum* and *C. baileyi* are more closely related to each other than they are to *C. muris*. The results of this study also suggest that *Cryptosporidium* parasites are not related to other coccidians at the SSU rRNA locus. This observation is in agreement with the results of previous studies (2, 10, 26). Extensive characterization at other genetic loci will be needed to determine the origin of *Cryptosporidium* parasites.

The results of phylogenetic analyses also indicated that there is significant intraspecies diversity in the genus *Cryptosporidium*. The three human *C. parvum* isolates differ from the three bovine isolates in four regions of the SSU rRNA gene. This difference between the two genotypes of *C. parvum* is in agreement with the presence of two genotypes in other genes observed by us and by other workers (3, 5, 6, 22, 25, 28, 29, 38, 40). Similarly, the *Cryptosporidium* isolate from guinea pigs, *C. wairi*, also differs from the bovine isolates in four regions, two of which are the same polymorphic regions that differ in the human and bovine genotypes. Because the evolutionary distance between *C. wairi* and the two *C. parvum* genotypes is much smaller than the evolutionary distances between other *Cryptosporidium* spp., *C. wairi* might be considered another genotype of *C. parvum* except for its biological characteristics, such as host specificity. A difference between the human and bovine genotypes of *C. parvum* at nucleotides 689 to 699 has also been observed recently (21). Using a partial SSU rRNA gene sequence, another group of workers has also identified a new *C. parvum* genotype (5, 7). The new genotype sequence (ICP), however, is identical to the sequence of our *C. muris* bovine isolate.

Sequence variation in the SSU rRNA gene was also observed in *C. muris* and *C. serpentis*. The SSU rRNA gene sequence of the *C. muris* bovine isolate is different from the sequences of the *C. muris* Bactrian camel isolate and the *C. muris* rock hyrax isolate, whereas the latter two are similar to each other. Interestingly, both the Bactrian camel isolate and the rock hyrax isolate infect young mice, whereas the *C. muris* bovine isolate does not infect these animals. Similarly, there are small differences in the SSU rRNA gene of *C. serpentis*. Two snake isolates differed in minor ways from two lizard isolates. It has been suggested previously that *C. serpentis* is a multispecies complex consisting of at least five species (36). Our results suggest that there may be some intraspecies biological differences among these *Cryptosporidium* parasites.

In addition to further refining the taxonomic relationships among *Cryptosporidium* parasites, the results of our sequence analysis allowed us to develop a species- and strain-specific diagnostic tool, which is urgently needed for monitoring the presence of the pathogens in the environment. This PCR-RFLP technique amplifies the sequences of all *Cryptosporidium* parasites, but species and genotypes can be identified by digesting the secondary PCR product with *SspI* and *VspI*, respectively. Previously, two PCR-RFLP techniques based on the SSU rRNA gene were developed to differentiate *C. parvum* from other *Cryptosporidium* parasites (1, 16). One technique (16) used conserved sequences for primers and therefore prob-

ably amplified the SSU rRNA genes of all eukaryotic organisms. The other technique (1) used an erroneous sequence (4) as the primer, which reduced the efficiency of amplification and made interpretation of data difficult.

In summary, the results of this study confirmed biological observations that the genus *Cryptosporidium* comprises multiple species. Based on the SSU rRNA gene sequence, the four species used in this study form two clades. Further studies with *C. meleagridis*, *C. felis*, and *C. nesorum* are needed to obtain a more complete phylogenetic profile. The data which we obtained contradict the results of a recent study (35) and are supported by host specificity data and other biological characteristics. In addition to redefining the species structure of the genus, the results of this study facilitated the development of species- and strain-specific diagnostic tools that are needed for monitoring the safety of drinking water.

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