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PRESENCE OF DOUBLE-STRANDED RNAs IN HUMAN AND CALF ISOLATES OF *CRYPTOSPORIDIUM PARVUM*

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ABSTRACT: We examined the occurrence of 2 virus-like double-stranded (ds)RNAs in human and calf isolates of *Cryptosporidium parvum* senso latu and other microorganisms, including 7 other members of the genus. A total of 32 isolates of *C. parvum,* 16 from humans (5 from acquired immune deficiency syndrome patients) and 16 from calves, were analyzed. Ethidium bromide staining, or Northern blot analysis, or reverse transcription/polymerase chain reaction, or all 3 methods, revealed that both genotype 1 and genotype 2 isolates of *C. parvum* possessed these dsRNAs. No other *Cryptosporidium* spp. or other organisms examined possessed these dsRNAs. Comparison analysis of partial cDNA sequences of dsRNAs from human and calf isolates revealed a high degree of similarity (>92% and >93% identical nucleotides for large and small dsRNAs, respectively). Slight, consistent differences in nucleotide sequences could be seen at select sites and were associated with an isolate being either genotype 1 or 2. Because of the widespread distribution of the dsRNAs, the similarity of these molecules between isolates, and high host specificity, these nucleic acids may prove to represent species-specific molecular markers for *C. parvum.* Evidence also suggests that the dsRNA can be utilized for molecular genotyping of *C. parvum.*

Human isolates of *Cryptosporidium parvum* are known to exist in at least 2 distinct groups (Morgan et al., 1998; Spano et al., 1998; Sulaiman et al., 1998; Widmer, 1998). The first group is found only in humans (genotype 1 or type H), whereas the second exists in both humans and many other animals (genotype 2 or type C). No definitive evidence yet exists that genetic exchange occurs between the 2 genotypes, at least on a consistent basis (Bonnin et al., 1996; Awad-El-Kariem et al., 1998; Morgan et al., 1998; Spano et al., 1998; Widmer et al., 1998). This suggests the possibility of 2 distinct species or clonal lines and different transmission cycles among ''*C. parvum*'' senso lato isolates infecting humans (Peng et al., 1997) and supports the hypothesis that human cryptosporidiosis is not always a zoonotic disease (Casemore and Jackson, 1984; Hojlyng et al., 1985; Awad-El-Kariem et al., 1993).

Recently, a high number of 2 extrachromosomal virus-like double-stranded (ds)RNAs were detected in oocysts of several calf isolates of *C. parvum* (Khramtsov et al., 1997). Clones of cDNAs for both dsRNAs from the KSU-1 isolate were generated and sequenced, and each was found to contain a single large open reading frame (ORF). The deduced protein sequence of the large dsRNA (L-dsRNA) possessed motifs characteristic of RNA-dependent RNA polymerases (RDRP). The function of the putative protein encoded by the small dsRNA (S-dsRNA) remains unknown. Crude oocyst homogenates were also found to contain a viral RDRP activity responsible for the replication of the dsRNAs (Khramtsov and Upton, 1998). The importance of these molecules for the parasite and its presence in human isolates with different genotypes are as yet unknown. However, because dsRNAs are common in many parasitic protozoa (Patterson, 1990; Wang and Wang, 1991), and some are known to modulate virulence of pathogenic fungi (Nuss and Koltin, 1990), we have continued to investigate the biology and occurrence of these dsRNAs in both human and animal isolates of *C. parvum.*

MATERIALS AND METHODS

Microorganisms and oocyst purification

A list of all parasites and their sources are presented in Table I. Oocysts of different *Cryptosporidium* spp. were purified from feces by CsCl gradient centrifugation and surface sterilized with 10% Clorox as described previously (Upton et al., 1994). The number and purity of oocysts were determined by microscopic analysis. Other microorganisms were either used without additional purification or were kindly provided by numerous scientists (see Table I).

Nucleic acid extraction

Purified parasites or feces were suspended in phosphate-buffered saline and then washed 3 times by low-speed centrifugation. Samples were subjected to 4 freeze–thaw cycles in lysis buffer (100 mM NaCl, 20 mM EDTA, 20 mM Tris-HCl, pH 7.5, 0.5% sodium dodecyl sulfate) followed by incubation at 56 C for 2 hr with 0.5 mg/ml of proteinase K. After extraction with phenol–chloroform, total nucleic acids were precipitated with ethanol.

Genotypic analysis

Genetic differences were detected by analyzing restriction fragment length polymorphisms (RFLP) in polymerase chain reaction (PCR)-amplified DNA fragments of 3 genetic loci, i.e., the polythreonine protein gene (polyT) with primers cry44 and cry39 (Carraway et al., 1997), the thrombospondin-related adhesive protein gene (TRAP-C2) (Peng et al., 1997; Sulaiman et al., 1998), and the heat-shock 70 protein gene (hsp70).

A 795-nucleotide (nt) fragment of hsp70 (coordinates from 1,574 nt to 2,368 nt, GenBank accession number U11761) was amplified in each isolate as described previously (Khramtsov et al., 1995). Each fragment was digested with the restriction endonuclease *Sau*3AI followed by agarose gel electrophoresis to resolve the DNA fragments. Sequences of 385 nt (coordinates from 1,616 nt to 2,000 nt) were determined using the *fmol* DNA Cycle Sequencing System (Promega Inc., Madison, Wisconsin).

Detection of dsRNAs by reverse transcription PCR (RT-PCR) and sequence analysis

RT-PCR was performed as described by Khramtsov et al. (1997) with primers for L-dsRNA (LV5: 5′-CCGAGTTGATGACTATAAATC, 46–

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TABLE I. Microorganisms examined for the presence of *Cryptosporidium parvum* dsRNAs.

Microorganism	Stage	Designation	Isolate origin: Original host	Isolate origin: Most recent passage	Locality (year isolated)
Cryptosporidium parvum					
Genotype 1	Oocysts	212458L*	Human	N/A	Missouri (1997)
	Oocysts	610542J*	Human	N/A	California (1997)
	Oocysts	HM2:93	Human	N/A	Wisconsin (1993)
	Oocysts	HM3:93*	Human	N/A	Wisconsin (1993)
	Oocysts	HM5:93*	Human	N/A	Wisconsin (1993)
	Oocysts	HM6:93*	Human	N/A	Wisconsin (1993)
	Oocysts	HGA8:97	Human	N/A	Georgia (1997)
	Oocysts	HGA5:95	Human	N/A	Georgia (1995)
	Oocysts	HFL6:95	Human	N/A	Florida (1995)
	Oocysts	H120	Human	N/A	Perth, West Australia (1998)
	Oocysts	H121	Human	N/A	Perth, West Australia (1998)
	Oocysts	H123	Human	N/A	Perth, West Australia (1998)
	Oocysts	H125	Human	N/A	Perth, West Australia (1998)
Genotype 2	Oocysts	6HMA1	Human	N/A	Maine (1993)
	Oocysts	H122	Human	N/A	Perth, West Australia (1998)
	Oocysts	H124	Human	N/A	Serpentine, West Australia (1998)
	Oocysts	GCH1†	Human	Calf	Massachusetts (1991)
	Oocysts	TAMU‡	Human	Calf	Texas (1994)
	Oocysts	KSU-1	Calf	Calf	Kansas (1987)
	Oocysts	KSU-4	Calf	Calf	Kansas (1999)
	Oocysts	Louisiana-1§	Calf	Calf	Louisiana (1997)
	Oocysts	Iowa A†	Calf	Calf	Iowa (mid-1980s)
	Oocysts	Iowa I‖	Calf	Calf	Iowa (mid-1980s)
	Oocysts	AuCp1	Calf	Calf	Alabama (1985)
	Oocysts	AuCp2	Calf	Calf	Florida (1997)
	Oocysts	UCP#	Calf	Calf	Iowa (mid-1980s)
	Oocysts	CISD	Calf	Mice	Texas (1996)
	Oocysts	C50	Calf	N/A	Perth, West Australia (1998)
	Oocysts	C51	Calf	N/A	Perth, West Australia (1998)
	Oocysts	C52	Calf	N/A	Perth, West Australia (1998)
	Oocysts	C53	Calf	N/A	Perth, West Australia (1998)
	Oocysts	C54	Calf	N/A	Perth, West Australia (1998)
Cryptosporidium baileyi	Oocysts	AuCb1	Chicken	Chicken	Alabama (1986)
Cryptosporidium meleagridis	Oocysts	None given	Turkey	Turkey	North Carolina (1996)
Cryptosporidium muris	Oocysts	108735¶	Rock hyrax	Mice	Virginia (1993)
Cryptosporidium serpentis	Oocysts	KSU-2	corn snake	N/A	Kansas (1996)
Cryptosporidum sp.	Oocysts	KSU-3**	Beef steer	N/A	Kansas (1999)
Cryptosporidium sp.	Oocysts	VS1742**	Dairy heifer	N/A	Idaho (1996)
Cryptosporidium sp.	Oocysts	95-400**	Beef steer	N/A	Idaho (1997)
Cryptosporidium sp.	Oocysts	None given††	Ostrich	N/A	Arizona (1995)
Cryptococcus neoformans	Cells	H99‡‡	N/A	N/A	North Carolina
Cyclospora cayetanensis	Oocysts	Albany§§	Human	N/A	New York (1996)
Eimeria bovis	Sporozoites	None given‖‖	Calf	Calf	Alabama (1996)
Escherichia coli	Cells	XL-1 blue	Unknown	In vitro	Unknown (unknown)
Giardia bovis	Cysts	None given	Calf	N/A	Kansas (1996)
Neospora caninum	Tachyzoites	NC-1‖‖	Dog	Cell culture	New York
Perkinus atlanticus	Zoospores	None given##	Clam	Cell culture	Portugal
Pneumocystis carinii	Trophozoites	None given††	Rat	N/A	North Carolina
Saccharomyces cerevisiae	Cells	CB001 pep4:: URA3, ura3, leu2, trp1¶¶	N/A	In vitro	Laboratory strain
S. cerevisiae	Cells	CG347; a, pet 8, met2, arg1, his7, met14, KIL-k***	N/A	In vitro	Laboratory strain
Sarcocystis miescheriana	Merozoites	None given†††	Pig	N/A	Georgia
Toxoplasma gondii	Tachyzoites	RH§§	Human	Cell culture	Laboratory strain

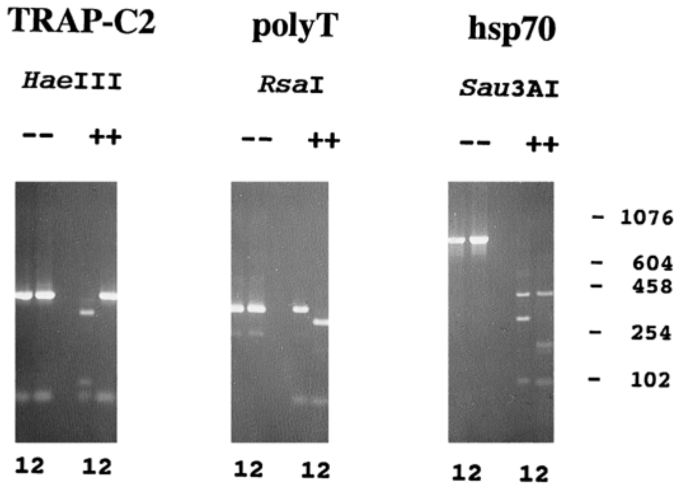

FIGURE 1. Genotype analysis of *Cryptosporidium parvum* isolates by PCR/RFLP. Agarose gel electrophoresis profiles of the PCR-amplified fragments of 3 genes (TRAP-C2, polyT, and hsp70) from representative human and calf isolates. PCR products were undigested (−) or digested (+) with restriction endonucleases. The isolates are indicated by numbers: 1 = human isolate 610542J; 2 = calf isolate KSU-1. Molecular size standards are in the right margin.

66 nt, and LV7: 5′-GGGACTAAGCCAGTTGGTATG, 246–226 nt) and S-dsRNA (SV7: 5′-CGGTATGTCGACTGACAAATC, 28–48 nt, and SV6: 5′-GCAGAAGGGTTCTATGATTC, 455–436 nt). Southern blot hybridization was performed using ^{32}P-labeled internal primers LVN: 5′-ATGAAGTTTGTCAATATCTATG, 134–155 nt, and SVN: 5′-ATGATTACAAGTTTTGAATCAATAG, 248–272 nt, for the L- and S-dsRNAs, respectively.

In other experiments RT-PCR products were obtained with primers for L-dsRNA (LVA: 5′-ctggatccGCTCAATTTTCGTCCAACG, 14–33 nt, or LVN: 5′-ctggatccATGAAGTTTGTCAATA, 134–149 nt, and LVB: 5′-gggtcgacTGCCTCATTCAACTTCTCTTCA, 361–340 nt) and for S-dsRNA (SVA: 5′-ctggatccTCGCATGGAGGTTCATGTAG, 192–211 nt, and SVB: 5′-gggtcgacTTGATCAAATCCATATCAGGTACTTTC, 495–469 nt) (lowercase represents nucleotide used for cloning). The amplified DNA was digested with *Bam*HI and *Sal*I restriction endonucleases and ligated into cloning vector pBluescript SK^{+}II (Stratagene, La Jolla, California), digested with the same enzymes, and used for transformation. The inserts of recombinant plasmid were sequenced. Coordinates of the primers are according to the GenBank accession numbers U95995 for L-dsRNA and U95996 for S-dsRNA (Khramtsov et al., 1997).

Northern blot analysis and other molecular biology techniques

Detection of *C. parvum* dsRNAs by Northern blot analysis was as described (Khramtsov and Upton, 1998). Southern blot analysis, plasmid DNA purification, ^{32}P-labeling of oligonucleotide, and other procedures were as described in Sambrook et al. (1989).

RESULTS

Genotypes of *C. parvum* isolates

Genetic analysis of *C. parvum* isolates was performed by PCR/RFLP (Fig. 1). To examine genotypes of *C. parvum,* 2 independent loci (polyT and TRAP-C2) were analyzed as published previously (Carraway et al., 1997; Peng et al., 1997). In the case of TRAP-C2, we utilized RFLP (Sulaiman et al., 1998) rather than sequencing (Peng et al., 1997) to detect differences between genotypes 1 and 2. We found that all isolates from calves, as well as 3 from humans (6HMA1, H122, and H124), were genotype 2, whereas the remaining 13 human isolates were genotype 1. Some of these human isolates have been genotyped by other investigators, and our results are in precise agreement with the previous analysis (Peng et al., 1997; Widmer et al., 1998).

We also chose to examine a portion of the hsp70 gene from each isolate in an attempt to determine whether this gene also possessed polymorphisms useful for genotype analysis. The 795-nt PCR-amplified fragments of hsp70 from human and calf isolates were subjected to digestion with the restriction endonucleases *Dra*I, *Hae*III, *Hin*fI, *Msp*I, *Rsa*I, and *Sau*3AI in order to search for polymorphic restriction sites. Two different electrophoretic profiles were seen on agarose gels after resolving the fragments following digestion with *Sau*3AI (Fig. 1). No differences were detected with any of the other restriction endonucleases (data not shown). All calf isolates, and 3 human isolates (6HMA1, H122, and H124) carried the same pattern, whereas 13 other human isolates possessed a second profile. Sequences of amplified fragments of hsp70 from human and calf isolates were determined (data not shown), and comparative analysis revealed only a single nucleotide substitution. Hsp70 from 13 human isolates had AATC (absence of the *Sau*3AI site), whereas all calf isolates and 3 human isolates (6HMA1, H122, and H124) had GATC (presence of the *Sau*3AI site) in position 1,667–1,670 nt (coordinates according to GenBank accession number U11761; Khramtsov et al., 1995). This demonstrates that at least 2 alleles of hsp70 are present among *C. parvum* isolates, one that tends to be associated with genotype 1 and the second associated with genotype 2.

←

* AIDS patient.
† Obtained through the NIH AIDS-reagent program.
‡ Although originally isolated from a human, the individual in question is thought to have been infected while attending a necropsy of an infected foal.
§ Purchased from Waterborne Technologies, New Orleans, Louisiana.
‖ Purchased from Pleasant Hill Farms, Troy, Idaho. The 2 Iowa isolates have been separated geographically (Arizona and Idaho) for over 10 yr.
Oocysts of the UCP (Ungar *Cryptosporidium parvum*) isolate were generously provided by Dr. Joseph Crabb, Immunocell, Inc. They were originally obtained in March 1990 from Dr. B. L. P. Ungar, who acquired them from Dr. Ron Fayer at the U.S. Department of Agriculture in Beltsville. This isolate was originally derived from the Iowa isolate.
¶ Passage 16 through juvenile mice but originally obtained from a rock hyrax at the National Park Zoo in Virginia.
** Large abomasal species in cattle. The VS1742 and 95-400 isolates were provided courtesy Dr. Bruce Anderson, Caldwell, Idaho.
†† Courtesy Dr. F. Lozano-Alarcon, Arizona Veterinary Diagnostic Laboratory.
‡‡ Courtesy Dr. J. R. Perfect, Duke University.
§§ Courtesy Dr. J. S. Keithly, New York State Department of Health.
‖‖ Courtesy Dr. David S. Lindsay, Auburn University.
Courtesy Dr. F. O. Perkins, Virginia Institute of Marine Sciences.
¶¶ Laboratory strain, originally from Dr. Elizabeth Jones, Carnegie Mellon University.
*** Courtesy Dr. M. Resnick, NIEHS.
††† Courtesy Dr. A. K. Prestwood, University of Georgia.

Detection of dsRNAs

Two ethidium bromide staining bands with approximate sizes of 1.7×10^3 nt and 1.4×10^3 nt were identified on agarose gels after resolution of total nucleic acid extracts of 2×10^7 oocysts from all calf isolates of *C. parvum* (Fig. 2A). In contrast, these bands were absent in samples from the same number of oocysts from 4 other *Cryptosporidium* species. Both bands were resistant to the presence of low concentration (0.1 μg/ml) RNase A but sensitive to high concentrations (10 μg/ml). These bands were still present after treatment of the sample in a high ionic strength buffer (0.3 M NaCl) but disappeared after incubation with RNase A in low ionic strength buffer (0.01 M NaCl), indicating that these fragments were dsRNAs (Khramtsov et al., 1997). Northern blot hybridization with ^{32}P-labeled cDNAs of L- and S-dsRNAs revealed positive signals only in samples from *C. parvum* isolates (Fig. 2B, C).

Because it was possible that the dsRNAs may have been present at such low concentrations in non-*C. parvum* species that they were not detected by staining with ethidium bromide or Northern blot hybridization, RT-PCR with primers designed according to cDNA sequences of L- and S-dsRNAs were used. Specific fragments were amplified and identified only in samples from *C. parvum* isolates (Fig. 3A, C). The specificity of RT-PCR was confirmed by positive hybridizations of amplified products with ^{32}P-labeled internal primers (Fig. 3B, D). RT-PCR was the only method employed in the detection of dsRNAs in the 16 human isolates of *C. parvum* as insufficient numbers of oocysts were available for ethidium bromide staining and Northern blot analysis (data not shown). Double-stranded RNAs were detected in all human and calf isolates of *C. parvum* but not in any other member of the genus or in any of the other microorganisms listed in Table I.

Similarity and differences in the sequences of dsRNAs

The detection of dsRNA in all *C. parvum* isolates by RT-PCR suggested that the sequences of dsRNAs from both human and calf isolates, and among genotypes, are similar. To confirm this and to examine the relationship between dsRNA from different isolates, the cDNA sequences of 306 nt of the L-dsRNA and 257 nt of the S-dsRNA were determined for 5 calf (genotype 2) and 7 human (5 with genotype 1; 2 with genotype 2) isolates. Comparative analysis (Fig. 4) revealed a high degree of similarity between isolates. A comparison of the nucleotide sequences of each isolate with KSU-1 revealed a similarity ranging from 92% to 99% for L-dsRNA and from 93% to 99% for the S-dsRNA. The majority of nucleotide substitutions (65% for L-dsRNA and 61% for S-dsRNA) occur in the third position of the codons, preserving the amino acid sequence. Although heterogeneity was present throughout all analyzed sequences, it was possible to separate the sequences unambiguously into 2 groups. These groups could be established on the basis of 15 and 10 nucleotide changes (double underlined in Fig. 4) for the L- and S-dsRNA, respectively. Phylogenetic analysis using cluster or topological algorithms confirmed the visual observations (data not shown). The first group is composed of sequences from all calf and human isolates typed as genotype 2, whereas the second group consists of genotype 1 human isolates.

In the case of 2 human isolates (HGA8:97 and H125), the

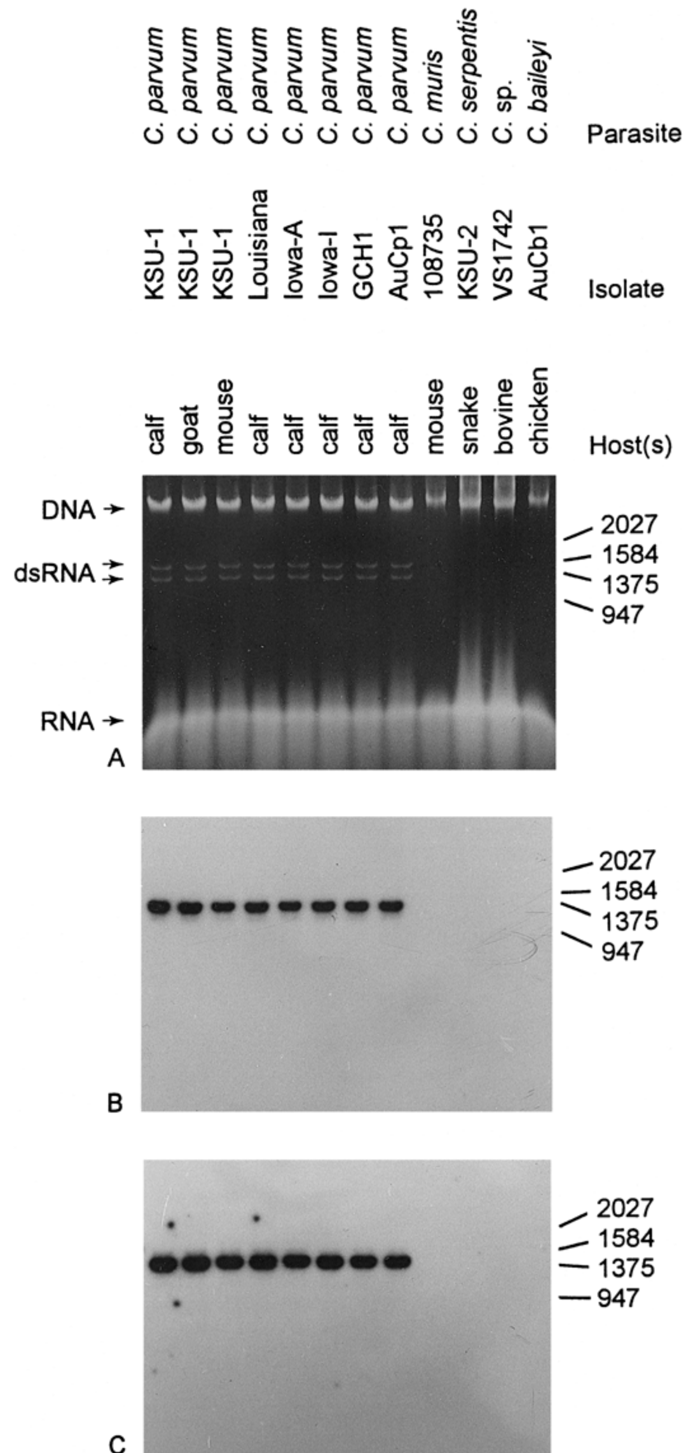

FIGURE 2. Detection of dsRNAs in *Cryptosporidium parvum* isolates by staining with ethidium bromide (**A**) and following Northern blot hybridization (**B, C**). In B and C, 2 parallel gels with identical samples of nucleic acids treated with 0.1 μg/ml RNase A were run and the nucleic acids transferred onto Nytran sheets. The autoradiograms show hybridization filters with ^{32}P-labeled cDNA fragments of L- (B) and S-dsRNAs (C), respectively. Molecular size standards are shown in the right margin.

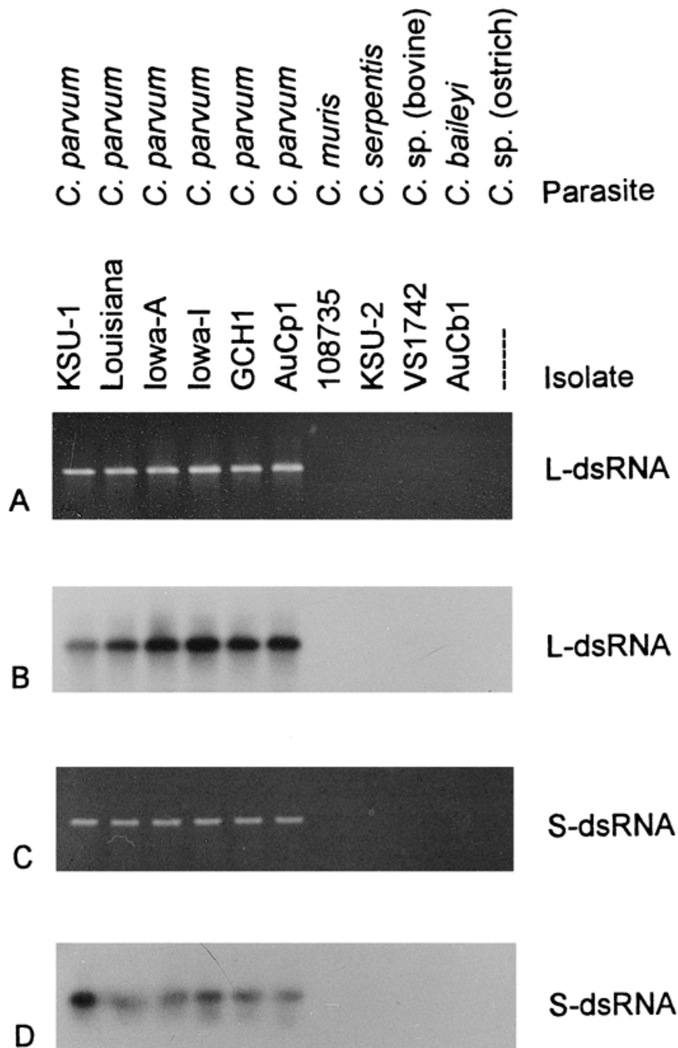

FIGURE 3. Detection of dsRNAs by RT-PCR (**A, C**), and with Southern blot analysis (**B, D**). Agarose gel electrophoresis of amplified products of nucleic acids from various *Cryptosporidium* isolates using primers specific to the L- (A) and S-dsRNA (C). Southern blots of the gels in panels A and C hybridized with ^{32}P-labeled internal oligonucleotides to L- (B) and S-dsRNA (D).

RT-PCR products for L-dsRNA were obtained only with primers LVN and LVB but not with LVA and LVB (see Material and Methods). It is possible that the LVA primer, located close to the 5′ end of the L-dsRNA of the KSU-1 isolate, has low sequence similarity with dsRNA from these isolates and did not anneal with targets. Alternatively, the L-dsRNAs of these 2 isolates may have degraded and were shorter than in other isolates.

DISCUSSION

The present study has revealed that the dsRNAs in *C. parvum* occur in numerous human and calf isolates but not in other members of the genus or other microorganisms. They appear to be persistent and widespread in the U.S. and in Australian populations of *C. parvum* and will probably be found in a wide variety of isolates wordwide. The occurrence of these molecules in isolates from 5 acquired immune deficiency syndrome (AIDS) patients undergoing antiretroviral therapy also suggests that human immunodeficiency virus (HIV) medical treatments probably do little to affect the dsRNAs. Detection of dsRNAs in developmental stages during in vitro cultivation of *C. parvum* (Khramtsov et al., 1997) reveals that these molecules also occur in other stages of the life cycle other than sporozoites. In the case of the KSU-1 isolate, dsRNAs have been observed continually since their first detection in 1992 (data not shown) and were identified by Khramtsov et al. (1997) in oocysts passaged through 3 different hosts (calf, goat, mouse). No significant changes in amounts, or in the ratio between the relative levels of L- and S-dsRNAs per oocyst, have been observed in the KSU-1 isolate, and no apparent differences in the amounts of dsRNAs occur between isolates (data not shown). On the basis of widespread distribution, similarity, and high host specificity, the dsRNAs may represent a molecular marker for *C. parvum.*

The ubiquitous presence of dsRNA elements is not unique to *C. parvum.* It was suggested that all in vivo isolates of *Trichomonas vaginalis* may have dsRNA, although some isolates lose them during in vitro cultivation (Khoshnan and Alderete, 1994). A restricted distribution of dsRNAs among some strains of *Leishmania* in the Amazon River basin makes these elements potential epidemiologic markers (molecular tags) (Salinas et al., 1996). Strains of *Giardia lamblia* from several hosts, including humans, were found to carry dsRNAs (Miller et al., 1988). These molecules were detected in strains from the U.S., England, and Poland but not from isolates originating in Belgium and Israel (De Jonckheere and Gordts, 1987).

The detection of both the L- and S-dsRNAs together in similar amounts in different isolates suggests that they are not simply a mixture of 2 independent dsRNAs nor are they defective or satellite molecules. Rather, they appear dependent on each other and interdependent for normal maintenance. It has been suggested that the dsRNAs of protozoa and fungi originated from an ancient noninfectious virus or group of viruses (Bruenn, 1993). These viruses have coevolved with their hosts and successfully persisted and spread to their hosts due to highly efficient intracellular transmission (Ghabrial, 1998). Although as yet unknown, it is possible that the dsRNAs of *C. parvum* do not have extracellular routes of infection and are transmitted between cells only during cell division and cell fusion. It is likely, however, that replication of the dsRNAs in *C. parvum* is tightly regulated as unregulated synthesis of dsRNAs could potentially kill the parasite. Therefore, 1 potential method of killing *C. parvum* in the host may be to up-regulate dsRNA synthesis.

We consider the heterogeneity (1–8%) between sequences of dsRNAs from the different isolates to be only moderate. Normally, a high rate of mutation might be expected due to the low fidelity of RNA genome replication because of the lack of proofreading enzymes that guarantee the fidelity of DNA replication (Holland et al., 1982; Steinhauer et al., 1992). Most nucleotide substitutions were found in the third codon position and failed to result in changes in amino acids. This provides evidence for a protein-coding function and suggests that the ORFs of dsRNA may encode proteins.

The RT-PCR and sequence analysis described here allows the identification of 2 closely related groups of dsRNAs in *C. parvum* isolates. The first group is associated with genotype 1 and the second with genotype 2 isolates. The association of different

A

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KSU-1 (2) AAAATTTTCTTTCCGAGTTGATGACTATAAATCAAGGCTAACCACCTACTCTACGGTTACTAGTATTGACAGTTCGACCTGCGCGGTTCT 123
Iowa  (2) ..........................................................................................
C1    (2) ..........................................................................................
C2    (2) ..........................................................................................
C3    (2) ..............................................................A...........................
C4    (2) ..........................................................................................
C5    (2) ..........................................................................................
H1    (2) ..............A...........................................................................
H2    (2) ..............A...............G...A.......................................................
H3    (1) ..G...........A...........................................................................
H4    (1) ..G.......................................................................................
H5    (1) ..G...........A...........................................................................
H6    (1)
H7    (1)

                    !  !  !  !  !  !  !  !  !  !  !  !  !  !  !  !  !  !  !  !  !  !  !  !  !  !  !
KSU-1 (2) TTCTACCGAAATGAAGTTTGTCAATATCTATGAGATACAACGGTTTGATGGCCAACCTACTAGACATGGAATAGCTCCAAAGAAAATATT 213
Iowa  (2) ..........................................T...............................................
C1    (2) ..........................................................................................
C2    (2) ..............................................................................T...........
C3    (2) ............................................C.............................................
C4    (2) ..................C.......................................................................
C5    (2) ..........................................................................................
H1    (2) ....................................................................................G.....
H2    (2) ...............................................................G....................G.....
H3    (1) ......T...................................TC.A.......................C..T...........G.....
H4    (1) ..........................................T..........................C..T...........G.....
H5    (1) ......T...................................T..A.......................C..T...........G.....
H6    (1)                           ................T..........................C..T...........G.....
H7    (1)                           ................T..A.......................C..T...........G.....

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KSU-1 (2) TCGTTCCAAATACATACCAACTGGCTTAGTCCCAAGACTAAAGTATTGGCGAGATGTACCTTCTAGAGCTGAAATGTCTAAAAGGATAGG 303
Iowa  (2) C.....T............................................G..............G.......................
C1    (2) C.....T............................................G..............G.......................
C2    (2) C..................................................G................T.....................
C3    (2) C..................................................G..............G.......................
C4    (2) C.....T...................C........................G........C.....G.......................
C5    (2) C..C...............................................G..............G.......................
H1    (2) C..C............T.G....................G...........G..............GA......................
H2    (2) ...C............T......................G...........G..............G...............G.......
H3    (1) ......T..G..................A.A..T.A.T.......C.....C...A.T.....G..G..A....................
H4    (1) .........G..T...T.G...........A..T.A.T.......C.....T...A.T.....G..G..A....................
H5    (1) ...A..T..G......T...........A.A..T.A.T.......C.....C...A.T.....G..G..A....................
H6    (1) .........G..T...T.G.........A.A..T.A.T.......C.....T...A.T.....G..G..A....................
H7    (1) ...A..T..G......T...........A.A..T.A.TC......C.....C...A.T.....G..G..A....................

           !  !  !  !  !  !  !  !  !  !  !  !
KSU-1 (2) TAAATACTTTGAAGATGAATTCAAGTTTTATCCGAA                                                       339
Iowa  (2) ....................................
C1    (2) ...............C....................
C2    (2) ....................................
C3    (2) ....................................
C4    (2) ....................................
C5    (2) ...............C.................A.T
H1    (2) ....................................
H2    (2) ....................................
H3    (1) .................................TG.
H4    (1) .................................TG.
H5    (1) .................................TG.
H6    (1) .................................TG.
H7    (1) .................................TG.
```

FIGURE 4. Relationship between cDNA of L- (**A**) and S-dsRNA (**B**) from genotype 1 and genotype 2 isolates of *Cryptosporidium parvum.* Alignments of sequences from calf (C1–C5) and human (H1–H7) isolates were constucted by SeqAid program. Numbers on the right margin correspond to the complete sequences of dsRNA of the KSU-1 isolate (GenBank accession numbers U95995 for L-dsRNA and U95996 for S-dsRNA). Numbers in parentheses indicate genotype (1 or 2). Sequences of the Iowa isolate were extracted from the dbest database (AA397489 and AA420917) and represent single pass sequence only. Dots represent identical nucleotides and gap deletions. The first in-frame ATG (Khramtsov et al., 1997) codons are shown in bold. The putative initiation codon of L-dsRNA is only tentative and may actually be further upstream beyond the presented sequence. The first position of each codon is marked with an ! atop the KSU-1 sequence. Codons that may encode different amino

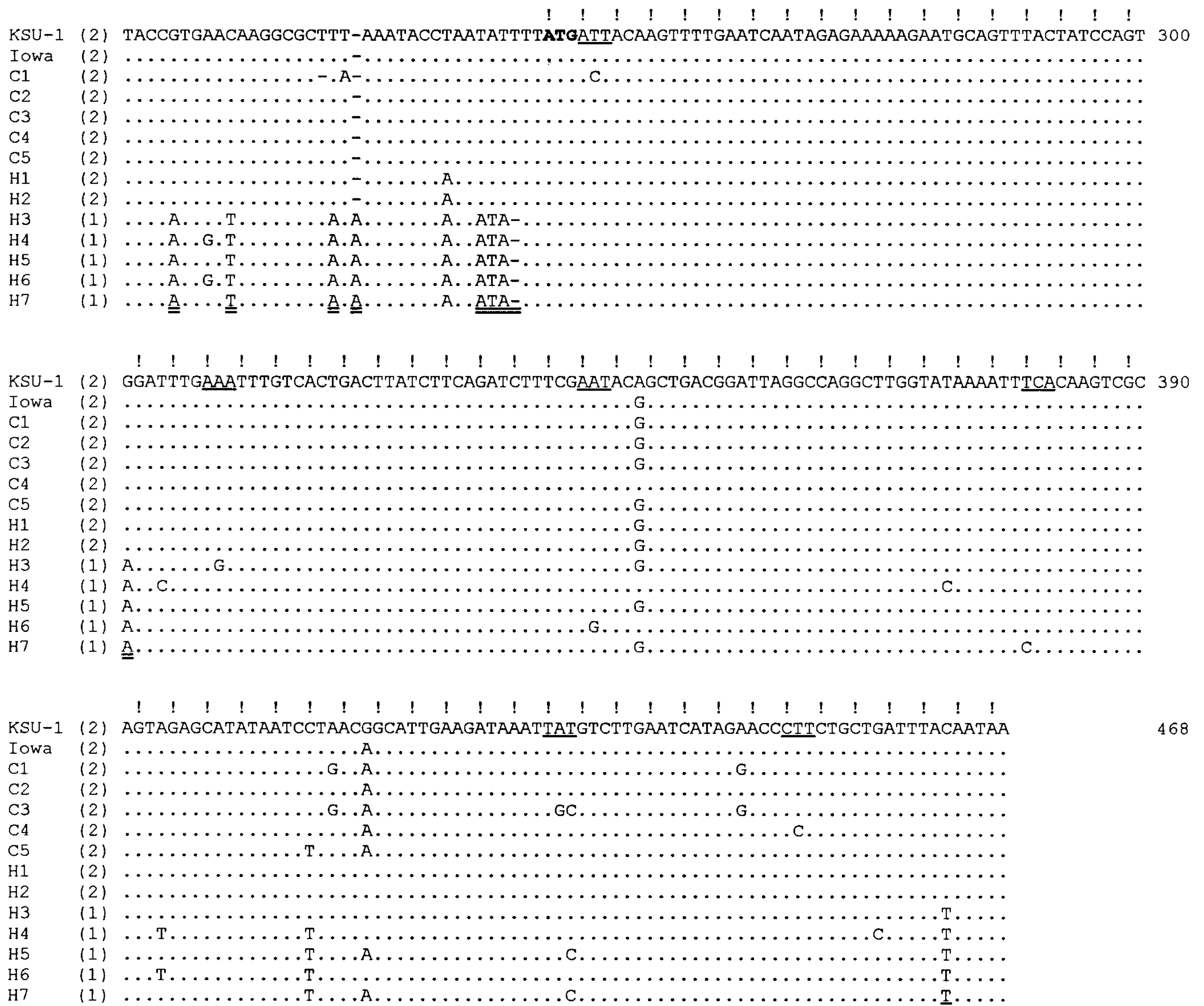

FIGURE 4. Continued.

dsRNAs with 2 genetically distinct *C. parvum* populations can be explained on the basis of the coevolution of virus-like dsRNAs and host cells (*C. parvum*). The evolution of RNA viruses in general, as well as dsRNA viruses, is unavoidably linked to host evolution (Widmer and Dooley, 1995; Domingo and Holland, 1997; Ghabrial, 1998). The population structure of *C. parvum* is under intensive investigation; however, the proposition that the distinct genotypes represent different species or different clonal lines has not been universally accepted. The data presented in this paper do not totally clarify this issue, but the finding of 2 groups of extrachromosomal dsRNAs associated with the different genotypes adds more evidence for the existence and divergence of at least 2 genetically distinct groups of *C. parvum.*

←

acids in the different isolates are underlined. Nucleotides that are similar in all sequences of genotype 1 but different from all genotype 2 sequences are double underlined. Nucleotide sequences reported in this paper are available in the GenBank database under the accession numbers AF169922 and AF169934, for L- and S-dsRNA of C1–AuCp1 isolate, respectively; AF169923 and AF169935, C2–AuCp2; AF169924 and AF169936, C3–Louisiana-1; AF169925 and AF169937, C4—TAMU; AF169926 and AF169938, C5–C50; AF169927 and AF169939, H1–6HMA1; AF169928 and AF169940, H2–H124; AF169929 and AF169941, H3–212458L; AF169930 and AF169942, H4–610542J; AF169931 and AF169943, H5–HM2:93; AF169932 and AF169944, H6–HGA8:97; AF169933 and AF169945, H7–H125.

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