Molecular Analysis of the 18S rRNA Gene of *Cryptosporidium serpentis* in a Wild-Caught Corn Snake (*Elaphe guttata guttata*) and a Five-Species Restriction Fragment Length Polymorphism-Based Assay That Can Additionally Discern *C. parvum* from *C. wrairi*

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An adult wild-caught corn snake (*Elaphe guttata guttata*) was presented for humane euthanasia and necropsy because of severe cryptosporidiosis. The animal was lethargic and >5% dehydrated but in good flesh. Gastric lavage was performed prior to euthanasia. Histopathologic findings included gastric mucosal hypertrophy and a hemorrhagic erosive gastritis. Numerous 5- to 7- μ m-diameter round extracellular organisms were associated with the mucosal hypertrophy. A PCR, acid-fast stains, Giemsa stains, and an enzyme immunoassay were all positive for *Cryptosporidium* spp. PCR and restriction fragment length polymorphism (RFLP) analysis on gastric lavage and gastric mucosal specimens, and subsequent sequencing of the 18S rRNA gene, enabled a distinct molecular characterization of the infecting organism as *Cryptosporidium serpentis*. Until recently, studies on snake *Cryptosporidium* have relied on host specificity and gross and histopathologic observations to identify the infecting species. A multiple alignment of our sequence against recently published sequences of the 18S rRNA gene of *C. serpentis* (GenBank accession no. AF093499, AF093500, and AF093501 [L. Xiao et al., unpublished data, 1998]) revealed 100% homology with the *C. serpentis* (Snake) sequence (AF093499) previously described by Xiao et al. An RFLP method to differentiate the five presently sequenced strains of *Cryptosporidium* at this locus was developed. This assay, which uses *Spe*I and *Ssp*I, complements a previously reported assay by additionally distinguishing the bovine strain of *Cryptosporidium* from *Cryptosporidium wrairi*.

Cryptosporidium is a 5- to 10- μ m round protozoal parasite that affects the digestive epithelium of a variety of animal species. More than 20 species of *Cryptosporidium* have been identified with questionable host specificity (8, 17). Recently, questions regarding identification of the organism to species level have been brought to the forefront for five reasons, as follows. (i) The organism has been identified as causing a fatal diarrhea in immune-compromised humans (6). (ii) There have been numerous water- and food-borne outbreaks of the disease in humans (22). (iii) There is no effective treatment for the disease. (iv) Water treatment protocols lack effective disinfection methods to rid drinking water of viable organisms (22, 27). (v) A variety of environmental sources may be contaminated with oocysts, and therefore, they may harbor a variety of different species of *Cryptosporidium* (11, 23). Identification of *Cryptosporidium* organisms to species level is important because species-specific pathogenicity to humans has not yet been determined. Until we are able to determine the specificity of the different species of *Cryptosporidium*, we cannot determine the epidemiological significance of its presence within environmental samples (i.e., understanding its importance and implementing control methods).

In snakes, the absence of an effective treatment for cryptosporidiosis necessitates euthanasia to eliminate patient suffering and prevent further spread of the parasite. Historically,

oocyst size and location in the host have been used in identification to species level (1a, 8, 16, 17, 21). However, a recent, sequence-specific PCR has been used to distinguish *Cryptosporidium parvum* from other species (4). Leng et al. (15) have described a PCR and restriction fragment length polymorphism (RFLP) method to analyze sequence variation of the 18S rRNA gene of *Cryptosporidium* spp. The high degree of sequence homology present in the 18S rRNA genes among different isolates and species of *Cryptosporidium* (3, 13, 14) allows the use of specific primers to amplify a wide range of *Cryptosporidium* species. Concomitant RFLP analysis is capable of exploiting any subtle differences found in these highly homologous nucleotide areas, provided restriction sites which enable differentiation exist.

In this study, two oligonucleotide primers, sequences 18SFwd (5'-AACCTggTTgATCCTgCCAg-3') and 18SRev (5'-TgATCCTTCTgCAGgTTCACCTA-3') (GenBank accession no. L16997) (2a, 17a) were used to amplify ca. 1,750-bp fragment of the 18S rRNA gene of *Cryptosporidium*. These primers target the 18S rRNA gene of all presently sequenced species of *Cryptosporidium*. This fragment was analyzed by RFLP and sequenced. We give the resulting complete nucleotide sequence of the 18S rRNA gene of *Cryptosporidium serpentis* (1,743 bp; GenBank accession no. AF151376) from *Elaphe guttata guttata* and align the sequence against other known *C. serpentis* sequences at this locus. Based on our results we suggest an RFLP-based assay capable of distinguishing five species of *Cryptosporidium* and of discerning bovine *C. parvum* from *Cryptosporidium wrairi*.

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MATERIALS AND METHODS

Sample collection and tissue preparation. Gastric lavage was performed prior to euthanasia, and the contents were collected in a 10-ml sterile tube. The animal was euthanized with an intracardiac injection of 80 mg of pentobarbital sodium. A complete necropsy was performed. The contents of the large intestine were collected in a 1.5-ml sterile microcentrifuge tube. The gastric and intestinal mucosae were gently scraped, and the contents were collected into 1.5-ml sterile microcentrifuge tubes. Tissues were formalin fixed and paraffin embedded, and slide preparations were stained with hematoxylin and eosin, as well as Giemsa stain (20), for histological examination.

Staining procedures. Acid-fast stains were applied to prepared slides (20). In brief, slide preparations of the specimens were heat fixed and the slides were flooded with Carbol-fuchsin stain for 5 min over continuous heat. The slides were then washed with distilled water, followed by decolorization with acid alcohol. Methylene blue was chosen as the counterstain and applied for 1 min. The slides were then washed with distilled water, blotted dry, and examined for the presence of acid-fast organisms.

A *Cryptosporidium* genus-specific enzyme immunoassay (EIA) with a sensitivity of 20 ng of *Cryptosporidium*-specific antigen/ml was performed (ProSpect *Cryptosporidium* Microplate Assay; Alexon Inc., Sunnyvale, Calif.).

DNA extraction. *C. parvum* oocysts were provided by the National Institutes of Health AIDS Reagent Program and used as the positive control in this study. Control and snake specimens were treated identically as follows. Fifty microliters of sample was centrifuged at $16,000 \times g$ for 5 min, the supernatant was removed, and the pellet was suspended in 50 μ l of lysis buffer containing 1 M Tris-HCl, 0.5 M EDTA, 0.4 mg of proteinase K/ml, and 10% Triton X-100. The samples were frozen at -80°C for 5 min, then thawed at 75°C for 5 min. This freeze-thaw cycle was repeated a total of four times. Samples were then incubated at 75°C for 3 h and centrifuged at $16,000 \times g$ for 5 min, and the supernatant containing genomic DNA (gDNA) was collected. Ten microliters was used for PCR.

Molecular analysis. Ten microliters of gDNA was added to a PCR mixture to make a 50- μ l total reaction volume consisting of the following: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 4.0 mM MgCl_2 , 200 μ M each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP) (Gene Amp PCR Core Kit; Perkin-Elmer, Norwalk, Conn.), 2.5 U of *Taq* DNA polymerase (Perkin-Elmer), 1 μ M each primer, and sterile double-distilled H_2O . Twenty microliters of the ca. 1,750-bp product was resolved by electrophoresis on 1% agarose. The resolved product was then excised from the gel and purified (GlassMAX DNA Isolation Spin Cartridge System; Life Technologies, Rockville, Md.) to a volume of 40 μ l with 5 mM Tris-HCl (pH 8.3)–0.1 mM EDTA. Five microliters of the purified product was then used as the template in a second round of amplification. PCR parameters, including molar volumes of constituents and cycling conditions of both amplifications, were identical (except for the template volume) in each amplification. A GeneAmp PCR System 9600 Instrument System was used for cycling: 35 cycles of 94°C (30 s), 68°C (30 s), and 72°C (90 s), followed by a 10-min extension step at 72°C . The ca. 1,750-bp product from the reamplification was excised and purified to a volume of 40 μ l with 5 mM Tris-HCl (pH 8.3)–0.1 mM EDTA as above, and 10 μ l was digested with *Dra*I and *Vsp*I (Promega Corporation, Madison, Wis.) for 1 h at 37°C . Fragments were separated on agarose, visualized with ethidium bromide, and mapped. Negative controls containing water in place of gDNA as well as gDNA extracted from a negative snake were run concurrently.

The purified 1,750-bp reamplification product was cloned into pCR2.1 (TA Cloning Vector; Invitrogen, Carlsbad, Calif.) and transformed in INVaF⁺ competent cells (One Shot; Invitrogen). Ampicillin-resistant colonies were screened via restriction digestion with *Eco*RI (Promega) for 1 h at 37°C . Cells were grown overnight in Luria-Bertani broth, and the plasmid DNA was prepared for sequencing with the Stratagene (La Jolla, Calif.) Clearcut Mini-Prep Kit according to the manufacturer's instructions.

Originally, nonspecific annealing prevented direct sequencing from the 18SFwd and 18SRev primers used for PCR; therefore, automated sequencing (DNA Core Lab, University of Miami School of Medicine, Miami, Fla.) was initially primed from the M13 Forward (–20) primer in pCR2.1 (Invitrogen). Two more forward sequencing primers, GL2RFwd1 (5'-AAGACGGCCATgC ACCACCAC-3') and GL2RFwd2 (5'-ggCAGTTgCCTTAAgCACTC-3'), were designed to complete the sequencing. To confirm the fidelity of *Taq* during polymerization and the resulting nucleotide sequence, three amplicons were analyzed independently, and their sequences were compared.

Nucleotide sequence accession number. The nucleotide sequence of the 18S rRNA gene of *C. serpentis* from *E. guttata guttata* has been deposited in GenBank under accession no. AF151376.

RESULTS

Gross findings. An adult wild-caught corn snake was presented for elective euthanasia and necropsy following a diagnosis of chronic cryptosporidiosis. This diagnosis was based on positive acid-fast staining of a gastric lavage specimen. The animal was >5% dehydrated but in good flesh. Digesta were noted within the intestinal lumens. Approximately 0.5 ml of

TABLE 1. Results of acid-fast staining, EIA, and PCR test performed for detection of *Cryptosporidium* spp. on specimens collected from various locations in a corn snake (*E. guttata guttata*)

Specimen	Result from:		
	Acid-fast staining	EIA	PCR
Gastric lavage	+	+	+
Gastric mucosa	+	+	+
Large-intestine mucosa	–	+	–
Feces	–	+	–

excrement was collected. The gastric mucosa was mildly reddened and friable (possibly exacerbated in part by the gastric lavage). No other gross abnormalities were noted.

Histopathology. Gastric sections were characterized by extensive hypertrophy of mucus-secreting cells lining the mucosal surface. Numerous 5- to 7- μ m round extracellular organisms were associated with the hypertrophied cells. Giemsa stains on gastric tissue were positive for *Cryptosporidium*. Acid-fast staining and an enzyme-linked immunosorbent assay were positive for *Cryptosporidium* (Table 1). Organisms were positive for viability by trypan blue staining. Additionally, there were multiple foci of mixed inflammatory infiltrates in the areas that had the highest organism density. Inflammatory cells include heterophils, lymphocytes, plasma cells, and macrophages. Mild hemorrhage was also noted within areas of inflammation.

Other changes included diffuse vacuolar degeneration in the liver. The remaining tissues (heart, lung, kidney, ovary, gall bladder, spleen, pancreas, brain, eye, skin, fat, trachea, esophagus, tongue, and intestines) were unremarkable.

PCR, RFLP, and sequencing. Amplification of gDNA extracted from a gastric lavage specimen and gastric mucosal scrapings with 18SFwd and 18SRev yielded visible bands at approximately 1,750 bp. No bands were visible after the first amplification for negative controls, fecal samples, and large-intestine mucosal samples. Both bands and the areas corresponding to 1,750 bp from the fecal and large-intestine mucosal samples were excised and reamplified. This reamplification elicited a substantial increase in the brightness of the gastric lavage and gastric mucosal bands at 1,750 bp over the initial amplification. No bands were visible for negative controls, feces, or intestinal mucosa in the 1,750-bp regions even after reamplification. Digestion of the reamplified products from the gastric lavage and intestinal mucosa with *Vsp*I resulted in visible fragments of approximately 920 and 820 bp. *Dra*I did not cut the reamplification products (Fig. 1). These results are consistent with our sequence data showing a single *Vsp*I site beginning at position 918 and no sites for *Dra*I (Fig. 2).

Sequence analysis of the ca. 1,750-bp product amplified from gDNA extracted from both the gastric lavage and gastric mucosa revealed a high degree of nucleotide sequence homology to 18S rRNA genes of other *Cryptosporidium* species (Table 2). There is an *Ssp*I restriction site at position 223 (Fig. 3) of *C. serpentis*, which is not present at this position in *C. parvum*, *Cryptosporidium muris*, *C. wairi*, or *Cryptosporidium baileyi*. Recently, GenBank has accepted sequences of the 18S rRNA gene of *C. serpentis* (accession no. AF093499, AF093500, AF093501, and AF093502 [28]). A multiple alignment of our sequence against those of Xiao et al. (28) reveals that our *C. serpentis* (*guttata*) sequence is identical with the *C. serpentis* (snake) sequence of Xiao et al. (Table 3). Initially, a single amplicon was sequenced and revealed three apparent nucleotide discrepancies with the *C. serpentis* (snake) sequence of Xiao et al., at positions 998, 1156, and 1477. However, confirmation of this sequence by the subsequent analysis of three

FIG. 1. Electrophoretic profile of the RFLP analysis from *VspI* digestion of the 18S rRNA gene of *Cryptosporidium* spp. Lane 1, *C. parvum*; lane 3, *C. serpentis* from gastric mucosa; lane 5, *C. serpentis* from gastric lavage; lane 7, 100-bp DNA ladder (Gibco BRL). Lanes 2, 4, and 6 are empty. Arrows indicate previously reported digestion of *C. muris* 18S rRNA (15).

additional amplicons disputed these substitutions and produced three identical sequences, each of which was 100% homologous to the *C. serpentis* (snake) sequence of Xiao et al. An *SspI* restriction site discovered at position 223 of our sequence, a site which is not present at this position in *C. parvum*, *C. muris*, *C. wairi*, or *C. baileyi*, is conserved in all three sequences of Xiao et al. (28).

DISCUSSION

Clinically, *Cryptosporidium* was high on the list of differential diagnoses for this animal. The positive acid-fast staining and EIA results supported this diagnosis. The initial PCR to screen for *Cryptosporidium* spp. provided the most definitive evidence.

The histologic findings also were consistent with cryptosporidiosis, and these organisms are most likely *C. serpentis*, based on location (i.e., gastric). This is in agreement with previously reported data on *Cryptosporidium* in snakes (2, 5). The positive EIA results on the feces and the large-intestine mucosa were unexpected. These positive results were possibly from the passage of antigenic remnants rather than the actual presence of the organism (7, 9, 10, 12). Other possibilities which might explain the disparity between EIA and PCR include the inhibition of PCR by contaminants coextracted with the gDNA (19, 24) and the sensitivity of PCR. Although the limits of detection for the PCR were not determined in this case, previous studies utilizing primers targeting the 18S rRNA gene of *Cryptosporidium* have been capable of detecting as little as one oocyst by two rounds of amplification without subsequent hybridization (18). Addition of 10 to 20 µg of bovine serum albumin/ml to PCR mixtures, in an attempt to relieve any inhibition, had no effect.

The changes observed in the liver were most likely reflective of the anorexia observed clinically.

Our assumption that this organism was *C. serpentis* was not verifiable by the PCR and RFLP protocol previously described (3, 13, 14). In fact, Leng et al. (15) recently used PCR-RFLP to differentiate *C. parvum*, *C. muris*, and *C. baileyi*, and based on their choice of restriction enzymes, our initial evaluation of the genomic DNA extracted from gastric lavage and intestinal membranous scrapings with PCR-RFLP were suggestive of *C. muris* (Fig. 1). This was of particular interest considering the

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1  5' AACCTGGTGTGATCCTGCCAGTAGTCATATGCTGTCTCAAAGATTAAGCCATGCATGTCT
    18SFwd
61  AAGTATAAGCTTTTATACGGCGAACTGCGAATGGCTCATTAAACAGTTATAGTTTACT
121 TGATAATCAAACACTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGCGAAAAGGC
181 CCGACTTTTGGGAAGGTTGTATTTATTAGATAAAGAACCATAATTTTGGTGATTTCATA
    Ssp I
241  ATAACTTTACGGATCGCATCTCTGATGCGACATATCATTCAAGTTTCTGACCTATCAGCT
301  TTAGACGCTAGGGTATTGGCTACCGTGGCTATGACGGGTAACGGGGAATTAGGGTTCGA
361  TTCCGAGAGGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGGCGCGCAAA
421  TTACCAATCTGACACAGGGAGGTAGTGACAAGAAATAACAATACAGGCGCTAACGGTC
481  TTGTAATTGGAATGAGTGAAGTATAAACCCCTTTACAAGTATCAATTGGAGGCAAGTCT
541  GGTGCCAGCAGCCGCGTAATTCAGCTCCAATAGCGTATATTAAGTTGTTGCAGTTAA
601  AAAGCTCGTAGTTGGATTCTGTTGTATTTTATAATATTATTAAGGTAATATTATATAT
    Ssp I      Ssp I
661  ATCAACATCTCTCCTATTATATTTTTTAATATATAGGAAATTTTACTTTGAGAAATTAGA
721  GTGCTTAAAGCAGGCAACTGCCTTGAATACTCCAGCATGGAATAATAAGTAAGGACTTT
    GL2RFwd2 site
781  GTCTTCTTGTGTTGTTCTAGGATAAAAGTAAATGGTTAATAGGGACAGTTGGGGCATTGC
841  TATTTAACAGTCAGAGGTGAAATCTTAGATTGTTTAAAGACGAAGTACTGCGAAGCAT
901  TTGCCAAGGATGTTTTCTTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATA
    Vsp I
961  CCGTCGTAGTCTTAACCATAAACTATGCCGACTAGAGATTGGAGGTTGTTCTTACTCCT
1021 TCAGCACCTTATGAGAAATCAAAGTCTTTGGGTTCTGGGGGAGTATGGTCGCAAGGCTG
1081 AAACCTAAAGGAATTGACGGAAGGGCACCACAGGAGTGAGCGCTCGCGCTTAATTTGAC
1141 TCAACACGGGAAACTCACCAGGTCAGACATAGGAAGGATTGACAGATTGATAGCTCTT
1201 TCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGAGTGATTGTCTGGT
    GL2RFwd1 site
1261 TAATTCGGTTAACGAACGAGACCTTAACCTGCTAAATAGATAATAAAATTTATTTTTTT
1321 ATTTTATCTTCTTAGAGGACTTTGCGTGTCTAACGCGAGGAAGTTGAGGCAATAACAG
1381 GTCTGTGATGCCCTTAGATGTCCTGGGCCGACGCGGCTACACTGATGCATCCAGCGAG
1441 TATATATCTCTGTTTCGAAGGAAATGGTAAATCTTGATGATGATCGATGATGGGATAG
1501 ATCATTGCAATTATTGATCTTTAACGAGGAATTCCTAGTAAGCGCAAGTCATCAGCTTGC
1561 GCTGATTACGTCCTCCCTGGCCTTTGTACACACCGCCGTCGCTCCTACCGATTGAGTGATCC
1621 GGTGAATAATTTCGGACCATGCTATAGTAGCAAAATACATAGTAAGGAAGATTTCGTAAGCC
1681 TTATCACTTAGAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGGAAGGA
    18SRev(reverse comp)
1741 TCA 3'

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FIG. 2. *C. serpentis* (*guttata*) (AF151376) 18S rRNA gene (1,743 bp).

low mortality, despite the high morbidity, of snakes afflicted with cryptosporidiosis and especially given that their natural diet includes mice. Additionally, there remains some argument as to whether all *Cryptosporidium* organisms actually represent different strains of a single species (26). We chose to sequence the targeted fragment because there is a high degree of sequence homology in the 18S rRNA gene among different isolates and species of *Cryptosporidium*. Based on our findings, we conclude that the organism identified in this snake is

TABLE 2. Nucleotide sequence homology^a in the 18S rRNA gene for four species of *Cryptosporidium* to *C. serpentis* (*guttata*)

Organism	Accession no.	% Homology
<i>C. serpentis</i>	AF151376	100
<i>C. muris</i>	L19069	95
<i>C. parvum</i>	L16996	94
<i>C. baileyi</i>	L19068	94
<i>C. wairi</i>	U11440	94

^a With BLAST (1).

FIG. 3. Schematic representation showing the resulting fragments of an *SspI*-*SpeI* digestion of the 18S rRNA gene in *Cryptosporidium* spp.

C. serpentis. Further investigation of available 18S rRNA gene sequences for *Cryptosporidium* spp. showed that coupling digestion by *SspI* with *SpeI* instead of *VspI* enables the differentiation of *C. parvum* (bovine) from *C. wrairi*. An *SpeI* restriction site at position 889 bp of the 18S rRNA gene of *C. wrairi* is not present at this locus in any isolate of *C. parvum* sequenced to date.

Conclusion. Identification of *Cryptosporidium* organisms to species level is necessary in order to achieve a complete understanding of the significance of these organisms as environmental contaminants. Further investigation is needed to develop an assay that is capable of identifying *Cryptosporidium* to species level. Significant steps toward this goal have been made. The initial discovery by Leng et al. (15) of combining PCR and enzymatic digestion of the 18S rRNA gene with *VspI* and *DraI* allowed identification of three species of *Cryptosporidium*: *C. parvum*, *C. muris*, and *C. baileyi*. The use of *VspI* and *DraI*, however, fails to distinguish *C. serpentis* from *C. muris* because both contain the *VspI* recognition sequence at the same position on the 18S rRNA gene. Likewise, neither the use of *VspI* and *DraI* by Leng et al. nor the recent combination of *VspI* and *SspI* by Xiao et al. (28) can distinguish the bovine isolates of *C. parvum* from *C. wrairi*. After evaluation of our

sequence and all available *Cryptosporidium* 18S rRNA gene sequences, we determined that digestion of the 18S rRNA gene with the restriction enzymes *SspI* and *SpeI* enables the differentiation of the five presently sequenced species of *Cryptosporidium*: *C. parvum*, *C. muris*, *C. baileyi*, *C. wrairi*, and *C. serpentis* (Fig. 3) at this locus. Unlike the method of Xiao et al., the use of *SpeI* enables the distinction between *C. wrairi* and the bovine strains of *C. parvum*. Our work on the infectivity and viability of cryptosporidiosis is ongoing. Presently, we are targeting other loci for comparative analysis while characterizing the 18S rRNA genes of other species of *Cryptosporidium*.

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TABLE 3. Nucleotide substitutions and their positions on the 18S rRNA gene from multiple alignment of *C. serpentis* (*guttata*) versus three other strains of *C. serpentis*

Position from 5'	Nucleotide ^b in:			
	<i>C. serpentis</i> (<i>guttata</i>) (AF151376)	<i>C. serpentis</i> (Snake) (AF093499)	<i>C. serpentis</i> (Savanna monitor) (AF093500)	<i>C. serpentis</i> (Savanna monitor) (AF093501)
60	T	T	[C]	T
369	A	A	[G]	A
558	T	T	[C]	T
683	T	T	[C]	T
698	A	A	[G]	[G]
1674	G	G	[A]	[A]

^a With CLUSTAL W (25).

^b Substitutions are in brackets.

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