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Evidence Supporting Zoonotic Transmission of *Cryptosporidium* spp. in Wisconsin▽

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***Cryptosporidium hominis* and *Cryptosporidium parvum* are the primary species of *Cryptosporidium* that infect humans. *C. hominis* has an anthroponotic transmission cycle, while *C. parvum* is zoonotic, infecting cattle and other ruminants, in addition to humans. Most cryptosporidiosis outbreaks in the United States have been caused by *C. hominis*, and this species is often reported as the primary cause of cryptosporidiosis in this country. However, outbreaks account for only 10% of the overall cryptosporidiosis cases, and there are few data on the species that cause sporadic cases. The present study identified the species/genotypes and subgenotypes of *Cryptosporidium* in 49 cases of sporadic cryptosporidiosis in Wisconsin during the period from 2003 to 2005. The species/genotype of isolates was determined by PCR restriction fragment length polymorphism analysis of the 18S rRNA and *Cryptosporidium* oocyst wall protein genes. The *C. parvum* and *C. hominis* isolates were subgenotyped by sequence analysis of the GP60 gene. Forty-four of 49 isolates were identified as *C. parvum*, and 1 was identified as *C. hominis*. Of the remaining isolates, one was identified as being of the cervine genotype, one was identified as being a cervine genotype variant, and two were identified as being of a novel human genotype, previously reported as W17. Nine different subgenotypes were identified within the *C. parvum* species, and two of these were responsible for 60% of the cases. In this study we found that most sporadic cases of cryptosporidiosis in Wisconsin are caused by zoonotic *Cryptosporidium* species, indicating that zoonotic transmission could be more frequently associated with sporadic cases in the United States.**

Cryptosporidium causes cryptosporidiosis, a disease reported in more than 40 countries on six continents (6). There is no effective therapeutic treatment; and chronic infection can develop in immunocompromised persons, in whom diarrhea can become chronic and life-threatening. In particular, *Cryptosporidium* is a frequent cause of diarrhea in human immunodeficiency virus-infected and AIDS patients, in whom serious complications are associated with CD4 counts below 200 cells μl^{-1} (20).

Cryptosporidium parvum and *C. hominis* are the primary species infecting both immunocompetent and immunocompromised individuals worldwide (19, 34, 38). *C. parvum* infects cattle and other ruminants, in addition to humans, while *C. hominis* almost exclusively infects humans. *Cryptosporidium* can be transmitted directly, by contact with infected persons or animals, or indirectly, by consumption of contaminated food or water.

The highest incidence of cryptosporidiosis in the United States has been found in the upper Midwest states (11). In particular, Wisconsin was reported as having the highest incidence of cryptosporidiosis for each year from 1999 to 2002 (11). The Wisconsin city of Milwaukee also had the largest cryptosporidiosis outbreak in 1993, where more than 400,000 people were infected following contamination of the municipal water supply (15).

Geographic differences in the human incidence of *C. parvum* and *C. hominis* have been identified. For example, *C. parvum* is generally reported to be more common in the United Kingdom (8, 9, 16, 17), while *C. hominis* is reportedly more common in the United States (26, 34). To date, U.S. studies identifying the causative species of cryptosporidiosis have been limited to outbreaks and sporadic cases occurring in AIDS patients from urban areas (34). While it is interesting that most cryptosporidiosis outbreaks in the United States are caused by *C. hominis*, outbreaks have been estimated to account for only 10% of the overall number of cryptosporidiosis cases (5). The anthroponotic *C. hominis* species might also predominate in urban environments, where human contact is more frequent than contact with animals, such as cattle. Given the limitations of the current data for the United States, there is a need to characterize the *Cryptosporidium* species from sporadic cases that are not confined to urban areas.

We report on the molecular characterization of *Cryptosporidium* isolates from cases of sporadic cryptosporidiosis in Wisconsin during the period from 2003 to 2005. Our findings indicate that zoonotic rather than anthroponotic transmission is responsible for most sporadic cryptosporidiosis in that state.

MATERIALS AND METHODS

Isolates. A total of 49 human fecal samples (samples WH1 to WH49) previously shown to be positive for *Cryptosporidium* by a direct immunofluorescent assay (Merifluor; Meridian Biosciences, Cincinnati, Ohio) were analyzed to determine the *Cryptosporidium* species and genotype. The samples were collected from patients from 2003 to 2005 and were stored without preservative at −20°C prior to analysis. The patient's county of residence and the date of sample collection were also obtained for each sample (Table 1), with some exceptions. Information on the

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TABLE 1. Species, genotypes and subgenotypes of *Cryptosporidium* spp. from cases of human cryptosporidiosis in Wisconsin

Isolate identifier	Collection date (day-mo-yr)	County of residence[a]	Species/genotype[c]	Subgenotype[d]
WH1	26-Feb-2003	Rock	*C. parvum*	ND[e]
WH2	10-Jun-2003	Richland	*C. parvum*	IIaA15G2R1
WH3	22-Jul-2003	Waukesha	Cervine	ND
WH4	2-Aug-2003	Waukesha	*C. parvum*	IIaA16G2R1
WH5	5-Aug-2003	Rock	Cervine variant	ND
WH6	20-Aug-2003	Wood[b]	*C. parvum*	ND
WH7	20-Aug-2003	Wood	*C. parvum*	IIaA15G2R2
WH8	20-Aug-2003	Wood	*C. parvum*	ND
WH9	20-Aug-2003	Wood	*C. parvum*	IIaA15G2R2
WH10	20-Aug-2003	Richland	*C. parvum*	ND
WH11	4-Sep-2003	Wood	*C. parvum*	ND
WH12	4-Sep-2003	Wood	*C. parvum*	IIaA15G2R1
WH13	4-Sep-2003	Wood	*C. parvum*	IIaA17G4R2
WH14	4-Sep-2003	Wood	*C. parvum*	IIaA15G2R1
WH15	5-Sep-2003	Wood	*C. parvum*	IIaA15G2R2
WH16	15-Sep-2003	Waukesha	*C. parvum*	IIaA16G3R2
WH17	15-Sep-2003	Wood	*C. parvum*	ND
WH18	15-Sep-2003	Wood	*C. parvum*	IIaA15G2R2
WH19	16-Sep-2003	Wood	*C. parvum*	ND
WH20	16-Sep-2003	Wood	*C. parvum*	ND
WH21	16-Sep-2003	Wood	*C. parvum*	IIaA16G3R2
WH22	17-Sep-2003	Wood	*C. parvum*	IIaA15G2R1
WH23	24-Sep-2003	Rock	*C. parvum*	IIaA16G1R1
WH24	29-Sep-2003	Wood	*C. parvum*	IIaA15G2R2
WH25	30-Sep-2003	Wood	*C. parvum*	IIaA15G2R1
WH26	19-Nov-2003	Waukesha	*C. parvum*	IIaA17G2R2
WH27	18-Dec-2003	Rock	*C. parvum*	IIaA15G2R2
WH28	7-Jan-2004	Rock	*C. parvum*	IIaA17G2R1
WH29	24-Mar-2004	Rock	*C. parvum*	IIaA18G2R1
WH30	29-Apr-2004	Manitowoc	*C. parvum*	IIaA17G2R2
WH31	14-May-2004	Green	*C. parvum*	IIaA16G3R2
WH32	18-May-2004	Oneida	*C. hominis*	IbA10G2
WH33	19-Jul-2004	Rock	W17	ND
WH34	3-Aug-2004	Rock	*C. parvum*	ND
WH35	6-Aug-2004	Manitowoc	*C. parvum*	IIaA16G3R2
WH36	22-Aug-2004	Rock	*C. parvum*	ND
WH37	17-Sep-2004	Rock	W17	ND
WH38	23-Sep-2004	Jefferson	*C. parvum*	IIaA15G2R2
WH39	27-Sep-2004	Manitowoc	*C. parvum*	IIaA15G2R2
WH40	2-Oct-2004	Manitowoc	*C. parvum*	IIaA15G2R1
WH41	11-Oct-2004	Walworth	*C. parvum*	IIaA15G2R2
WH42	13-Oct-2004	Walworth	*C. parvum*	ND
WH43	20-Oct-2004	Rock	*C. parvum*	ND
WH44	21-Oct-2004	Rock	*C. parvum*	ND
WH45	1-Nov-2004	Illinois	*C. parvum*	IIaA15G2R2
WH46	9-Jan-2005	Rock	*C. parvum*	IIaA15G2R1
WH47	8-Feb-2005	Illinois	*C. parvum*	IIaA17G2R1
WH48	10-May-2005	Rock	*C. parvum*	ND
WH49	18-May-2005	Rock	*C. parvum*	IIaA15G2R2

[a] Patient county of residence. For WH45 and WH47, the patients resided in the state of Illinois.

[b] Patient county of residence information was not available for samples from a clinic in Wood County. Isolates from this clinic were identified by the county of collection (i.e., Wood County) rather than by the county of patient residence.

[c] Species and genotypes were assigned following PCR-RFLP analysis of the 18S rRNA and COWP genes and, when necessary, sequence analysis of the 18S rRNA gene.

[d] Subgenotypes were assigned following sequencing and phylogenetic analysis of the GP60 gene.

[e] ND, not possible to determine the subgenotype.

patient's county of residence was not available for samples obtained from a clinic in Wood County, Wisconsin. Samples from this clinic were identified as originating from Wood County; however, the patients may not have resided in that county.

DNA extraction. DNA was extracted from fecal samples by alkaline digestion and phenol-chloroform extraction and was purified by using a QIAamp DNA stool mini kit (QIAGEN, Valencia, CA), as described previously (25). Briefly, 66.6 μl of 1 M KOH and 18.6 μl of 1 M dithiothreitol were added to a 1.5-ml centrifuge tube containing 100 to 200 μl of stool sample. The samples were incubated at 65°C for 15 min, neutralized with 8.6 μl of 25% HCl, and buffered with 160 μl of 2 M Tris-HCl (pH 8.3). DNA was extracted with 250 μl of phenol-chloroform-isoamyl alcohol (Invitrogen, Carlsbad, CA), mixed, and centrifuged (IEC Micromax centrifuge) at 3,330 × g for 5 min. The supernatant was removed to a 2.0-ml Eppendorf tube containing 1.0 ml of buffer ASL from the QIAamp DNA stool mini kit (QIAGEN). The DNA was further purified, in accordance with the manufacturer's instructions, and stored at −20°C until it was required.

Species/genotype identification. *Cryptosporidium* species and genotypes were identified by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the 18S rRNA and *Cryptosporidium* oocyst wall protein (COWP) genes. A fragment of the 18S rRNA gene was amplified by nested PCR, as described previously (30). The primary PCR, performed with primers 18SFor1 (5′-TTCT AGAGCTAATACATGCG-3′) and 18SRev1 (5′-CCCATTTCCTTCGAAACA GGA-3′), amplified a 1,300-bp fragment. The primary PCR mixtures contained 1 μl of template DNA, 1× PCR buffer (Promega, Madison, WI), 3 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate (dNTP), 1 μM each primer, 2.5 U of *Taq* DNA polymerase, and 2 μl of nonacetylated bovine serum albumin (BSA; 10 mg/ml; New England Biolabs, Beverly, MA) in a 50-μl reaction volume. Primary PCR cycling conditions consisted of an initial denaturation of 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 59°C for 45 s, and 72°C for 60 s, with a final extension of 72°C for 7 min. The secondary PCR, performed with primers 18SFor2 (5′-GGAAGGGTTGTATTTATTAGATAAAG-3′) and 18SRev2 (5′-AAGGAGTAAGGAACAACCTCCA-3′), amplified an 830-bp fragment within the primary PCR product. The reaction conditions were similar to those described above for the primary PCR, with the exception that 1 μl of the primary PCR product was used as the template and the $MgCl_2$ concentration was 1.5 mM. Cycling conditions for the secondary PCR consisted of 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 58°C for 90 s, and 72°C for 2 min. Secondary products of the expected size were digested with VspI and SspI (New England Biolabs) at 37°C overnight. The digested products were separated by electrophoresis, stained with ethidium bromide, and visualized under UV transillumination. Species assignment was made by comparing the RFLP profiles to the known profiles reported in the literature. In cases in which the profiles were ambiguous, sequencing was performed. The secondary PCR products were sequenced in both directions by using primers18sFor2 and 18sRev2. Sequencing was performed on an ABI 3730 DNA analyzer (Iowa State University, Ames).

A fragment of the COWP gene was amplified by a nested PCR protocol (24, 31). A 769-bp fragment of the COWP gene was amplified in a primary reaction with primers BCOWPF (5′-ACCGCTTCTCAACAACCATCTTGTCCTC-3′) and BCOWPR (5′-CGCACCTGTTCCCACTCAATGTAAACCC-3′). Amplification was performed in 25-μl volumes with 2.5 μl of template DNA, 1× PCR buffer (Promega), 1.5 mM $MgCl_2$, 250 μM each dNTP, 10 pmol of each primer, 1.5 U of *Taq* DNA polymerase, and 1 μl nonacetylated BSA (10 mg/ml; New England Biolabs). Cycling conditions consisted of an initial denaturation of 95°C for 3 min, followed by 30 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min, with a final extension of 72°C for 10 min. An internal 553-bp fragment was amplified from the primary PCR product in a secondary reaction with primers Cry9 (5′-GGACTGAAATACAGGCATTATCTTG-3′) and Cry15 (5′-GTAGA TAATGGAAGAGATTGTG-3′). The PCR amplification was performed in 50-μl volumes with 2.5 μl of the primary PCR product, 1× PCR buffer (Promega), 1.5 mM $MgCl_2$, 250 μM each dNTP, 10 pmol of each primer, 1 U of *Taq* DNA polymerase, and 2 μl of nonacetylated BSA (10 mg/ml; New England Biolabs). Cycling conditions for the secondary PCR consisted of an initial denaturation of 95°C for 3 min, followed by 30 cycles of 94°C for 50 s, 55°C for 30 s, and 72°C for 50 s, followed by a final extension of 72°C for 10 min. Secondary products of the expected size were digested with the restriction enzyme RsaI (New England Biolabs) at 37°C for 4 h (31). The digested products were separated by electrophoresis in a 3.2% agarose gel. Species assignment was made by comparing the RFLP profiles to the known profiles reported in the literature.

Subgenotyping. Isolates identified to the species level were further subgenotyped by sequence analysis of the GP60 gene (1). A primary product of approximately 900 bp was amplified with primers AL 3531 (5′-ATAGTCTCCGCTGT ATTC-3′) and AL 3535 (5′-GGAAGGAACGATGTATCT-3′). Amplifications were performed in 50-μl volumes with 1 μl of template DNA, 1× PCR buffer (Promega), 3 mM $MgCl_2$, 200 μM each dNTP, 200 nM each primer, 2.5 U of *Taq* DNA polymerase, and 2 μl of nonacetylated BSA (10 mg/ml; New England Biolabs). Primary PCR cycling conditions consisted of an initial denaturation of 95°C for 3 min, followed by 35 cycles of 94°C for 45 s, 59°C for 45 s, and 72°C for 1 min, with a final extension of 72°C for 10 min. An internal fragment of

approximately 870 bp was amplified from the primary PCR product in a secondary reaction with primers AL 3532 (5′-TCCGCTGTATTCTCAGCC-3′) and AL 3534 (5′-GCAGAGGAACCAGCATC-3′). The reaction conditions were similar to those described above for the primary PCR step, with the exception that 2 μl of the primary product was used as the template. The cycling conditions were also similar to those used for the primary PCR, with the exception that the annealing temperature was 50°C. Secondary products of the expected size were purified (Wizard SV; Promega) and sequenced in both directions with primers AL 3532 and AL 3534. Sequencing was performed on an ABI 3730 DNA analyzer (Iowa State University). The GP60 gene sequences were aligned by using the ClustalX program (35), and evolutionary distances were measured by using the Kimura two-parameter model to compute pairwise distances, with confidence determined from 1,000 bootstrap replications (14). Subtypes within GP60 allele families were determined on the basis of the number of TCA (A), TCG (G), and ACATCA (R) repeats in the microsatellite region, in accordance with the nomenclature described previously (33). For example, a sequence with 15 TCA, 2 TCG, and 2 ACATCA repeats was assigned A15G2R2.

Nucleotide sequence accession numbers. The 18S rRNA gene nucleotide sequences were deposited in GenBank under accession numbers DQ640638 to DQ640640. The GP60 gene nucleotide sequences were deposited in GenBank under accession numbers DQ640629 to DQ640637.

RESULTS

The species, genotypes, and subgenotypes of the *Cryptosporidium* strains isolated from cases of human cryptosporidiosis in Wisconsin from 2003 to 2005 are presented in Table 1.

Species/genotype identification. A *Cryptosporidium* sp. was detected in 49 samples by nested PCR amplification of the 18S rRNA gene. The majority of isolates (44/49) were identified as *C. parvum* by RFLP analysis of the product amplified with VspI and SspI (Fig. 1). This species assignment was further supported by PCR-RFLP analysis of the COWP gene. One isolate (isolate WH32) was identified as *C. hominis* by PCR-RFLP analysis of the 18S rRNA and COWP genes.

The RFLP patterns of the 18S rRNA genes from four isolates (isolates WH3, WH5, WH33, and WH37) did not match the profiles of known *Cryptosporidium* spp. The PCR products from these isolates were sequenced and compared with known sequences in GenBank by using the BLASTN algorithm. Isolates WH33 and WH37 were identical to *Cryptosporidium* genotype W17 deposited in GenBank (GenBank accession number AY737573 [13]). WH3 was identical to cervine genotype sequences (GenBank accession numbers AY737592 [13], AJ849465, and AF442484 [4]), while WH5 was a variant of the cervine genotype that differed by an AT deletion at positions 689 and 690 of the *C. parvum* 18S rRNA gene (GenBank accession number AF164102 [2]) (Fig. 2). The COWP gene locus of isolates WH3, WH5, WH33, and WH37 could not be amplified.

Subgenotyping. Subgenotyping by sequence analysis of the GP60 gene was successful for 31/49 isolates. The GP60 gene could not be amplified from the non-*C. parvum*/*C. hominis* isolates (isolates WH3, WH5, WH33, and WH37). Phylogenetic analysis placed all *C. parvum* isolates into a single allelic group (allelic group IIa), while *C. hominis* was placed in a separate group (allelic group Ib) (Fig. 3). Nine *C. parvum* subgenotypes were identified within allelic group IIa. The two most frequently occurring subgenotypes (subgenotypes IIaA15G2R2 and IIaA15G2R1) accounted for 60% of the *C. parvum* isolates. The *C. hominis* subgenotype was identified as IbA10G2.

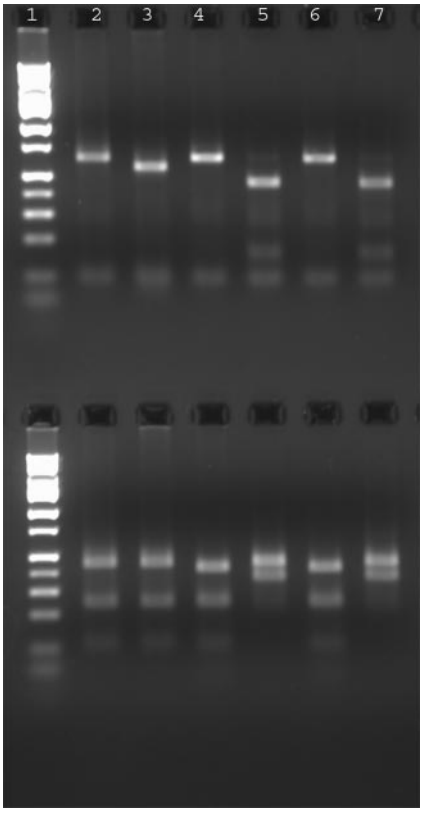

FIG. 1. Differentiation of *Cryptosporidium* by RFLP analysis of a PCR-amplified fragment of the 18S rRNA gene. Secondary PCR products were digested with VspI (upper panel) or SspI (lower panel). Lane 1, molecular weight markers; lane 2, *C. parvum*; lane 3, *C. hominis*; lane 4, W17 (WH33); lane 5, cervine variant genotype (WH5); lane 6, W17 (WH37); lane 7, cervine genotype (WH3).

DISCUSSION

We identified *C. parvum* as the primary cause of sporadic cryptosporidiosis in Wisconsin and found that it accounted for 90% of the cases examined from 2003 to 2005. The presence of *C. parvum* in humans is indicative of zoonotic transmission, with cattle and other ruminants being the likely sources. The significance of cattle in the zoonotic transmission of *Cryptosporidium* was previously shown in the United Kingdom during the 2001 foot-and-mouth disease outbreak. The animal contact restrictions put in place during that outbreak resulted in a significant reduction in the number of cases of human cryptosporidiosis caused by *C. parvum* (12).

The role of cattle in the zoonotic transmission of *C. parvum* in the region is further supported by GP60 subgenotyping data. Five of nine subgenotypes in the present study (Wisconsin) were previously reported in cattle in Michigan (25), while four of nine subgenotypes were reported in calves in Ontario, Canada (37). Subgenotype IIaA15G2R2, the most common subgenotype in Wisconsin, was also the most frequently occurring subgenotype in Michigan (25). Subgenotype IIaA15G2R2 was not found in calves in Ontario (37), although it was associated with a human case. That subgenotype does not appear to occur frequently in other regions of the world. IIaA15G2R1, a subgenotype that has frequently been reported worldwide, was the

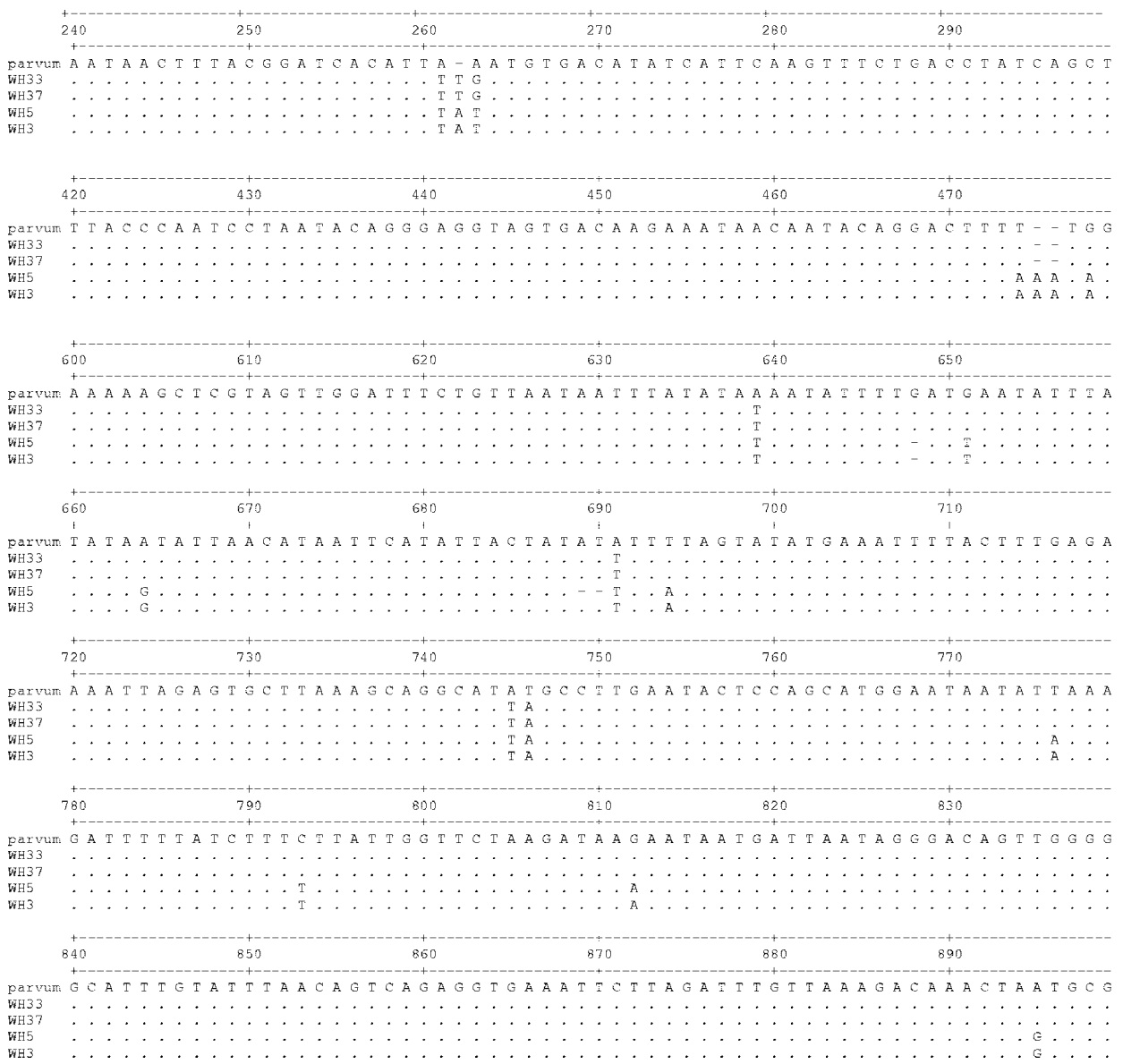

FIG. 2. Variable regions in aligned 18S rRNA sequences from the *C. parvum* Iowa isolate (GenBank accession no. AF164102 [2]) and isolates WH33 (W17), WH37 (W17), WH3 (cervine), and WH5 (cervine variant) from the present study. The ruler measures the distance (in nucleotides) from the beginning of the 18S rRNA gene in the Iowa isolate.

second most common subgenotype in Wisconsin and Michigan (25) and the most common subgenotype in Ontario (37).

The two cases of the cervine genotype identified indicate that wild animals may also be a reservoir for cryptosporidiosis in Wisconsin. The cervine genotype was first reported in white-tailed deer in New York (27) and has since been identified in storm event water (13), wastewater (39), and captive lemurs (4). This genotype was also previously reported in humans; the first case was identified in British Columbia, Canada (21), and the second one was reported in Ontario (37). The cervine genotype variant has not previously been identified in humans but was identified in white-tailed deer (27) and storm event water (13). The epidemiological or clinical significance of the variant genotype is not yet known.

We identified a novel human genotype in two separate cases of cryptosporidiosis. This genotype shared 100% similarity with genotype W17, previously reported in storm event water in New York State (13). W17 has not previously been reported in humans or animals. Although the reservoir for this genotype is yet to be identified, it is likely to be a wild animal (13), as it has not been identified in previous extensive studies of domestic animals and humans.

Cervine, cervine variant, and W17 are novel genotypes that can be added to the growing list of unusual species and genotypes that have been infrequently reported in humans (3, 7, 10, 18, 22, 23, 28, 36, 38). A number of factors may explain the low incidences of unusual species and genotypes relative to the incidences of *C. parvum* and *C. hominis*. One factor may be poor adaptation to human hosts, which could result in infections less severe than those caused by *C. parvum* or *C. hominis*.

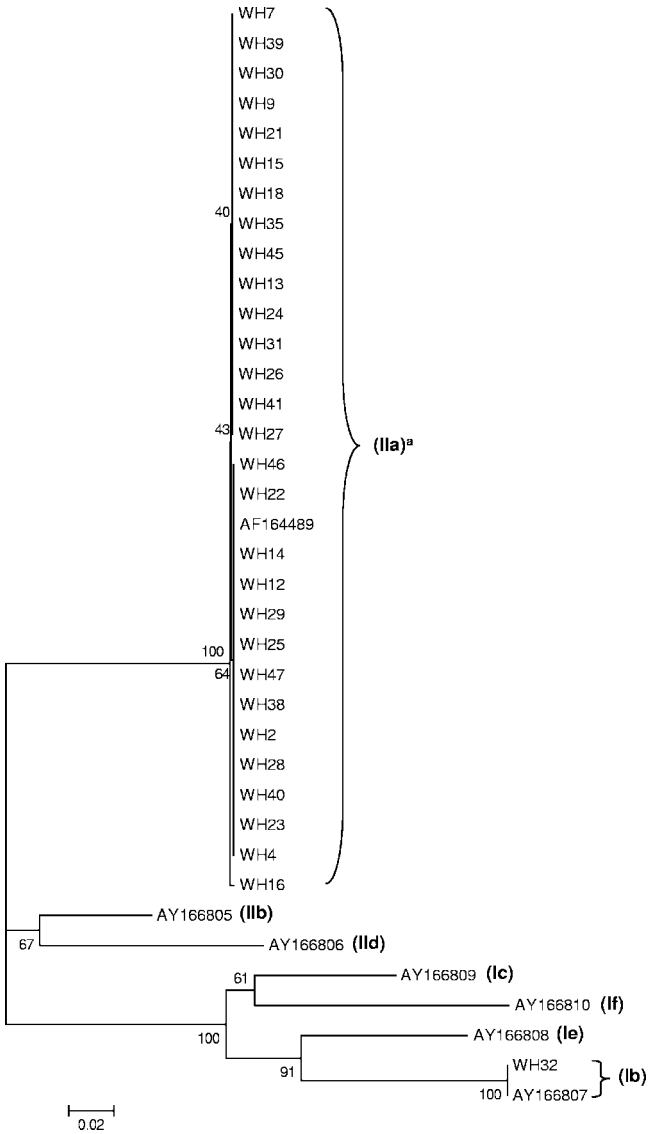

FIG. 3. Phylogenetic relationships at the *gp60* locus among sequences from this study and sequences previously deposited in GenBank. The sequence with GenBank accession no. AF164489 was previously reported by Strong et al. (32). The sequences with GenBank accession nos. AY166805 to AY166810 were previously reported by Alves et al. (1). [a], allelic group.

Infections of low severity may be underreported and would therefore be underrepresented in studies such as ours, which rely on visits to a physician. For cases reported to a physician, routine testing is not likely to identify isolates to the species or genotype level. Many public health laboratories in the United States still use detection methods that are only genus specific (29). The natural reservoir for unusual species and genotypes may also be important in explaining their relatively low frequencies in humans. In the case of the cervine, cervine variant, and W17 genotypes reported in the present study, transmission to humans from what is likely to be a wild animal reservoir may be less frequent than transmission from intensively farmed animals (*C. parvum*) or from other humans (*C. hominis* and *C. parvum*).

The low incidence of *C. hominis* would indicate that anthroponotic transmission does not contribute significantly to sporadic cryptosporidiosis in Wisconsin. *C. hominis* is often cited as the primary cause of cryptosporidiosis in the United States, a premise that is based largely on data from outbreaks and sporadic urban cases (26, 34). The rate of anthroponotic transmission is reported to be higher in urban areas. The specificity of our patient data was limited to the county of residence; therefore, we could not determine whether the population was rural or urban. However, it is significant that none of the isolates in the present study were collected from Milwaukee County, the county with the highest population density in Wisconsin. This absence of isolates from Milwaukee was most likely due to the relatively low prevalence of cryptosporidiosis in that county. While Milwaukee County contains 18% of the population of Wisconsin, data from the Bureau of Communicable Diseases, Wisconsin Division of Public Health, show that only 2 and 0.5% of cryptosporidiosis cases occurred in that county in 2004 and 2005, respectively (unpublished data). The low prevalence of *Cryptosporidium* in Milwaukee suggests that the controls put in place since the 1993 outbreak have been effective.

In this study we found that most sporadic cases of cryptosporidiosis in Wisconsin are caused by zoonotic *Cryptosporidium* species, indicating that zoonotic transmission could be more frequently associated with sporadic cases in the United States.

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