

Morphologic, Host Specificity, and Molecular Characterization of a Hungarian *Cryptosporidium meleagridis* Isolate

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Received 10 September 1999/Accepted 1 November 1999

This study was undertaken in order to characterize *Cryptosporidium meleagridis* isolated from a turkey in Hungary and to compare the morphologies, host specificities, organ locations, and small-subunit RNA (SSU rRNA) gene sequences of this organism and other *Cryptosporidium* species. The phenotypic differences between *C. meleagridis* and *Cryptosporidium parvum* Hungarian calf isolate (zoonotic genotype) oocysts were small, although they were statistically significant. Oocysts of *C. meleagridis* were successfully passaged in turkeys and were transmitted from turkeys to immunosuppressed mice and from mice to chickens. The location of *C. meleagridis* was the small intestine, like the location of *C. parvum*. A comparison of sequence data for the variable region of the SSU rRNA gene of *C. meleagridis* isolated from turkeys with other *Cryptosporidium* sequence data in the GenBank database revealed that the Hungarian *C. meleagridis* sequence is identical to a *C. meleagridis* sequence recently described for a North Carolina isolate. Thus, *C. meleagridis* is a distinct species that occurs worldwide and has a broad host range, like the *C. parvum* zoonotic strain (also called the calf or bovine strain) and *Cryptosporidium felis*. Because birds are susceptible to *C. meleagridis* and to some zoonotic strains of *C. parvum*, these animals may play an active role in contamination of surface waters not only with *Cryptosporidium baileyi* but also with *C. parvum*-like parasites.

Cryptosporidium parvum is a coccidian parasite that was recently recognized as an important intestinal pathogen of humans. Most *C. parvum* clinical infections are associated with a self-limiting diarrheal illness, but cryptosporidia can cause chronic, life-threatening disease in immunocompromised patients (7). In recent years there has been a dramatic increase in recognition of the importance of waterborne transmission of human cryptosporidiosis worldwide. In 1993, an outbreak in Milwaukee resulted in infection of more than 400,000 people and about 50 deaths (16). The lack of effective therapy for this disease complicates the control of human and animal cryptosporidiosis (7). In many countries, current drinking water regulations require that water intended for human consumption should not contain pathogenic organisms (8). However, little is known about the major source of oocyst contamination of surface and drinking waters and the sources of infection of human populations (14). Currently, PCR-based methods for monitoring water samples for *C. parvum* oocyst contamination are being developed. The possible existence of many *Cryptosporidium* species fostered the development of DNA techniques suitable for typing isolates. The commonly used techniques are PCR-restriction fragment length polymorphism analysis (2, 22, 23) and sequence analysis of taxonomically relevant loci (3, 24, 30, 42). However, it has been reported that some PCR methods cannot differentiate among *Cryptosporidium meleagridis* and various genotypes of *C. parvum*-like parasites (4).

Compared to the number of reported cases of infection by the other avian *Cryptosporidium* species, *Cryptosporidium*

baileyi, the number of reported cases of *C. meleagridis* infection in birds is low (34, 36). To date, only the morphology of putative *C. meleagridis* isolates (9, 10, 28, 32) and the morphology and infectious potential of *C. meleagridis* isolates for birds (20) and mammals (6) have been described. Because some bovine isolates of *C. parvum* are known to infect birds (19, 27, 39) and because *C. meleagridis* and *C. parvum* oocysts cannot be differentiated unequivocally on the basis of size or morphology, it is not certain that *C. meleagridis* and *C. parvum* were the species analyzed in the studies mentioned above. It has also been suggested that *C. meleagridis* might be identical or very closely related to *C. parvum*, which infects more than 100 species of mammals (4, 12). It was recently demonstrated by Xiao et al. that *C. meleagridis* could be distinguished from *C. parvum* on the basis of the small-subunit (SSU) rRNA gene sequence (42). However, in this study the researchers did not characterize the morphology or the biologic features of the United States isolate studied.

The goal of the present study was to characterize and determine the taxonomic status of a putative *C. meleagridis* strain isolated from a turkey in Hungary by combining the traditional methods of classification with a sequence similarity analysis of a variable region of the SSU rRNA gene by using a recently described fast and reliable typing method for *Cryptosporidium* species (3, 30).

MATERIALS AND METHODS

Parasites. Oocysts of *C. meleagridis* CMELH-1 were isolated from an 8-week-old turkey (strain 44 British United Turkeys BIG-6) in Hungary and were passaged in 1-week-old turkey poults of the same strain as described previously (37). Before infection, fecal samples were collected daily and examined to determine whether *Cryptosporidium* oocysts were present by using Sheather's sugar flotation method (29). Moderate oocyst shedding started on day 4 postinoculation and lasted for 8 to 10 days. Oocysts of *C. parvum* CPARH-1 were isolated from a 2-week-old calf in Hungary and were passaged in female C57BL/6N

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TABLE 1. Host ranges, locations, and morphometric characteristics of *Cryptosporidium* species that infect mammals, birds, and reptiles

Taxon	Host range ^a		Main location(s) ^c	Oocyst length (μm) ^d	Oocyst width (μm) ^d	Oocyst shape index ^d
	Group	Occurrence ^b				
<i>C. parvum</i> ^{e,f}	Mammals	F ^g	Small intestine	4.9 (4.5–5.4) ^h	4.4 (4.2–5.2)	1.1 (1.0–1.3)
	Birds	N ⁱ				
	Lower vertebrates	N				
<i>C. meleagridis</i> ^e	Mammals	F	Small intestine	5.0 (4.5–6.0)	4.4 (4.2–5.3)	1.1 (1.0–1.3)
	Birds	F				
	Lower vertebrates	N				
<i>C. wrairi</i> ^j	Mammals	F ^k	Small intestine	NA ^l	NA	NA
	Birds	N				
	Lower vertebrates	N				
<i>C. felis</i> ^j	Mammals	F	Small intestine	4.6 (3.2–5.1)	4.0 (3.0–4.0)	1.1 (1.0–1.2)
	Birds	ND				
	Lower vertebrates	ND				
<i>C. baileyi</i> ^j	Mammals	N	Cloaca, bursa, trachea	6.4 (5.6–7.5)	4.8 (4.8–5.7)	1.3 (1.1–1.4)
	Birds	F				
	Lower vertebrates	N				
<i>C. serpentis</i> ^j	Mammals	N	Stomach	6.2 (5.6–6.6)	5.3 (4.8–5.6)	1.2 (1.0–1.3)
	Birds	ND				
	Lower vertebrates	F				
<i>C. muris</i> ^j	Mammals	F	Stomach	7.7 (6.5–8.1)	5.2 (5.0–6.0)	1.5 (1.2–1.6)
	Birds	N				
	Lower vertebrates	N				

^a Data from references 1, 3, 7, 26, and 36.

^b F, found; N, not found; ND, no data.

^c Data from references 26 and 36.

^d Data from references 3, 18, 31, and 36.

^e Data from this study and previous studies.

^f *C. parvum* is not a uniform species. There are several genotypes (anthroponotic, zoonotic, canine, etc.) that have different host ranges, but the phenotypes are very similar (3).

^g The host range is narrow to wide, depending on genotype (e.g., anthroponotic versus zoonotic).

^h Mean (range)

ⁱ The zoonotic genotype is found in birds.

^j Data from previous studies.

^k The host range is narrow.

^l NA, not available. According to Chrisp et al. (5), "guinea pig cryptosporidia have essentially the same oocyst shape and size . . . as does *C. parvum*."

specific-pathogen-free mice (Charles River Laboratories, Wilmington, Mass.) by using the method of Healey et al. (15). Oocysts were collected and stored and inocula were prepared by using standard procedures (29).

Morphologic evaluation of oocysts. Oocysts of *C. meleagridis* and *C. parvum* that were less than 2 weeks old were purified by using discontinuous sucrose gradient centrifugation (38) and were washed in phosphate-buffered saline (pH 7.2) to remove potassium dichromate before measurements were obtained. Purified oocysts (40 oocysts of both isolates) were measured by using a magnification of $\times 1,250$ and Nomarski interference contrast microscopy. A statistical analysis was performed by using the InStat 2.0 program (GraphPad Software Inc., San Diego, Calif.). Mean values were compared by performing the Student *t* test. Differences were considered significant when *P* was <0.05 .

Host specificity and tissue location. Thirty female C57BL/6N pathogen-free mice (Charles River Laboratories) weighing 14 to 16 g were immunosuppressed with phosphated dexamethasone (15) and were divided into the following three groups: uninfected control mice, mice infected with *C. parvum* (ICP mice), and mice infected with *C. meleagridis* (ICM mice). The animals in the ICP and ICM mouse groups were inoculated intragastrically with 10^6 *C. parvum* oocysts and 10^6 *C. meleagridis* oocysts, respectively. Feed (Charles River Laboratories) and boiled water were available ad libitum. After inoculation, daily fecal samples were obtained from the mice and were examined by using Sheather's sugar flotation method. One-half of the animals were killed on day 10 postinoculation, and the entire digestive tract of each mouse was fixed in 10% buffered formalin and used for histopathology studies. Hematoxylin- and eosin-stained sections were prepared from the stomach, small intestine, and large intestine and were examined by light microscopy. The oocysts were collected and purified as described above. The total oocyst output of mice was determined from the pooled samples by the quantitative method based on the low sedimentation speed of oocysts (41).

Newly hatched Arbor Acres male chickens (Bábolna Co., Bábolna, Hungary) were housed in electrically heated wire bottom batteries with continuous illumination. Feed and boiled water were available ad libitum. The basal diet (Bábolna Co.) consisted of a commercial type of finisher ration that contained 16.6% crude protein and had been specially formulated to exclude anticoccidial agents, antioxidants, and antibiotics. When the birds were 7 days old, they were assigned to three groups of 10 on the basis of body weight (uninfected control, ICM, and ICP chickens) and were inoculated orally with 10^6 *C. meleagridis* oocysts or 10^6 *C.*

parvum oocysts isolated from mice. The experimental design and method were identical to the experimental design and method described above.

DNA extraction and PCR amplification. Purified *C. parvum* and *C. meleagridis* oocysts were harvested by centrifugation, and each pellet was resuspended in 200 μl of saline EDTA (10 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl; pH 7.5) containing 10 μl of 20% (wt/vol) sodium dodecyl sulfate and 10 μl of a 10-mg/ml solution of proteinase K. To release total genomic DNA, the samples were frozen (liquid nitrogen, 5 min) and thawed (75°C, 5 min) three times and then incubated at 58°C for 1 h. DNA was extracted with phenol-chloroform and was further purified with a Prep-A-Gene DNA purification kit as described by the manufacturer (Bio-Rad, Hercules, Calif.). *Cryptosporidium* genus-specific primers (CPBDIAGF and CPBDIAGR) were used to amplify the *Cryptosporidium* SSU rRNA variable region as described previously (17). Reactions were performed by using the GeneAmp 2400 PCR system (Perkin-Elmer, Foster City, Calif.). The following conditions were used for PCR: initial denaturation 95°C for 15 min; 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 1 min; and a final extension step consisting of 72°C for 9 min. Finally, the samples were cooled and kept at 4°C. PCR products were detected on ethidium bromide-stained 2% agarose gels (Gibco, Grand Island, N.Y.) by visualizing them with UV light.

DNA sequencing and sequence analysis. PCR products were purified by using the Prep-A-Gene DNA purification kit. Sequencing reactions were carried out with the Perkin-Elmer Big Dye kit, and the products were analyzed with a model ABI 310 genetic analyzer (Perkin-Elmer Biosystems, Foster City, Calif.). The partial sequences of SSU rRNA genes were manually aligned with the sequences of other cryptosporidia published previously by using the ARB program package (21).

Nucleotide sequence accession numbers. The sequences of the SSU rRNA gene diagnostic fragments of *C. parvum* CPARH-1 and *C. meleagridis* CMELH-1 have been deposited in the GenBank database under accession no. AJ242471 and AJ242472, respectively.

RESULTS AND DISCUSSION

Morphology. While the oocysts of *C. baileyi*, *Cryptosporidium muris*, and *Cryptosporidium serpentis* can be clearly distin-

<i>Cryptosporidium parvum</i> genotype 1	AAGCTCGTAG TTGATTCT GTTAATAATT TATATAAAAT ATTTTGATGA ATATTTATAT	60
<i>C. meleagridis</i>T.....T.....	59
<i>C. parvum</i> genotype 2	60
<i>C. wrairi</i>T.....-A.....	59
<i>C. parvum</i> genotype 1	AAATTTAACA TAATTCATAT TACTATTTTT TTTTTAGTA TATGAAATTT TACTTTGAGA	120
<i>C. meleagridis</i>AA...-...A.....	116
<i>C. parvum</i> genotype 2A.A-.....	117
<i>C. wrairi</i>A.A-.....	117
<i>C. parvum</i> genotype 1	AAATTAGAGT GCTTAAAGCA GGCATATGCC TTGAATACTC CAGCATGGAA TAATATTAAA	180
<i>C. meleagridis</i>	176
<i>C. parvum</i> genotype 2	177
<i>C. wrairi</i>	177
<i>C. parvum</i> genotype 1	GATTTTTATC TTTTTTATTG GTTCTAAGAT AAGAATAATG ATTAATAGGG ACAGTTGGGG	240
<i>C. meleagridis</i>C.....A.....	236
<i>C. parvum</i> genotype 2C.....	237
<i>C. wrairi</i>C.....	237
<i>C. parvum</i> genotype 1	GCATTTGTAT TTAACAGTCA GAGGTGAAAT TCTTAGATTT GTTAAAGACA AACTAATGCG	300
<i>C. meleagridis</i>	296
<i>C. parvum</i> genotype 2	297
<i>C. wrairi</i>G.....	297

FIG. 1. Alignment of the *Cryptosporidium* SSU rRNA gene diagnostic fragments obtained with primers CPBDIAGF and CPBDIAGR for *C. parvum* anthroponotic genotype 1, *C. meleagridis*, *C. parvum* zoonotic genotype 2, and *C. wrairi*. Only the first 300 columns of the alignments are shown, as the remaining columns were identical for all of the genotypes. Dashes indicate gaps, and dots indicate bases that are identical to the *C. parvum* genotype 1 bases. The GenBank accession numbers for the *C. parvum* genotype 1, *C. meleagridis*, *C. parvum* genotype 2, and *C. wrairi* sequences shown are L16997, AJ242472, AJ242471, and U11440, respectively. In the complete SSU rRNA coding sequence for the anthroponotic genotype of *C. parvum* (GenBank accession no. L16997), the region shown corresponds to positions 602 to 901.

guished from *C. meleagridis* oocysts, the oocysts of *Cryptosporidium felis*, *C. meleagridis*, *Cryptosporidium wrairi*, and various *C. parvum* genotypes are similar in terms of size and morphology (Table 1). The form and structure of the *C. parvum* and *C. meleagridis* oocysts examined in this study were identical to the form and structure of oocysts described by other workers (20, 40). We were not able to detect any significant morphologic differences between *C. parvum* and *C. meleagridis* oocysts in terms of oocyst wall morphology, the size or localization of the oocyst residuum, and the number or morphology of sporozoites. Like *C. felis* (31), the differences between the lengths and widths of the oocysts of *C. parvum* and the oocysts of *C. meleagridis* were slight, but they were significant (*C. parvum* oocysts were 5.0 ± 0.05 by 4.4 ± 0.07 μm , while *C. meleagridis* oocysts were 4.8 ± 0.02 by 4.2 ± 0.03 μm). However, the magnitude of the intraspecific variation in *C. parvum* isolates is similar to the magnitude (3) observed in this study. Thus, morphologic analysis alone cannot be used to differentiate *C. meleagridis* from the *Cryptosporidium* species mentioned above.

Host specificity and site of infection. In some previous studies, mild to moderate infections have been established in chickens following inoculation of *C. parvum* oocysts isolated from cattle (26). Other attempts to transmit various isolates of *C. parvum* to birds were unsuccessful (26, 36); nevertheless, anti-cryptosporidial antibodies were detected in the sera of the birds (25, 35), suggesting that an infection was established. Moreover, after infection with a Belgian bovine isolate of *C. parvum*, the total oocyst output of infected chickens was only 17% of the oocyst output of previously uninfected birds after oral challenge with *C. baileyi*, indicating that the parasite became established and that protective immunity developed (35). These findings suggest that strains of *C. parvum* differ with respect to host specificity. Recently, oocysts of *C. meleagridis* isolated from chickens proved to be infectious for several species of mammals, including mice, rats, rabbits, and cattle (6). In our study, the oocysts of *C. meleagridis* isolated from turkeys were successfully passaged in birds and were transmitted to immunosuppressed mice and vice versa. Similarly, the Hungarian bovine isolate of *C. parvum* passaged in mice was success-

fully transmitted to birds. In ICM and ICP mice, the prepatent periods (3 days), the patencies (until death between days 10 and 27 postinoculation), and the numbers of excreted oocysts (1.6×10^8 and 2×10^8 oocysts/animal) were almost identical. Oocyst shedding by ICM and ICP chickens started 3 days postinoculation and lasted until 16 and 12 days postinoculation, respectively. The total oocyst outputs of ICM and ICP birds were 7×10^6 and 3×10^6 oocysts/bird. The low oocyst output of ICM birds is consistent with previously described data (18). As observed previously (26, 36), we found that *C. meleagridis* and *C. parvum* mainly infected the small intestine.

SSU rRNA-based molecular typing. The sequence of the diagnostic fragment of the Hungarian bovine isolate *C. parvum* isolate CPARH-1 was identical to the sequences described for the *C. parvum* zoonotic genotype obtained from other geographic regions (30). After our *C. meleagridis* diagnostic SSU rRNA sequence for Hungarian isolate CMELH-1 was submitted to the GenBank database, another group submitted the complete sequence of the region coding for SSU rRNA for a North Carolina isolate of *C. meleagridis* (42) (GenBank accession no. AF112574). The overlapping regions of these two sequences are identical, suggesting that there may be very little genetic variation in the SSU rRNA region among geographically distinct isolates of this species. The sequence signature of the variable region of the SSU rRNA gene is unique (Fig. 1). Nevertheless, the high AT content of this region precludes the use of mutation-specific PCR and hybridization with specific probes; this leaves PCR followed by DNA sequencing as the most reliable technique for identifying species.

It was recently demonstrated that *C. meleagridis* could not be distinguished from *C. parvum* by simple diagnostic PCR assays (4). Because there are marked differences in the cross-transmission potentials of various *Cryptosporidium* species or genotypes (Table 1) and because morphological methods are unreliable, it is necessary to use a standardized molecular technique for species identification in this genus. As shown in this study, sequencing the variable region of the SSU rRNA (3, 30) may be a reliable typing (species identification) technique. Other genetic loci, especially those coding for proteins, may be lim-

ited to identification of only the zoonotic and anthroponotic *C. parvum* genotypes (30).

The prevalence of cryptosporidiosis and the number of *C. parvum*-like oocysts in the feces of some wild bird species can be high (14, 33). Our findings indicate that birds may serve not only as passive carriers, as suggested by others (11, 13), but also as active vectors that increase contamination of water with some *C. parvum*-like parasites and play a role in waterborne cryptosporidiosis outbreaks. However, accurate identification of bird isolates and studies of the host ranges of these isolates, including studies of the susceptibility of humans to *C. meleagridis*, will be necessary in order to estimate the relative importance of birds in the epidemiology of cryptosporidiosis in humans and other mammals.

ACKNOWLEDGMENTS

We thank Zsuzsanna Egyed and Jánosné Haluska for technical assistance.

The Netherlands Organization for Scientific Research (NWO-OTKA grant N-28783), the Hungarian Research Fund (OTKA grants F-14646 and T-26057), a Bolyai János Postdoctoral Research Fellowship (grant BO/00480/98), and the European Cooperation in the Field of Scientific and Technical Development (COST Action 820) provided financial support.

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