APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1999, p. 1455–1458 Vol. 65, No. 4
0099-2240/99/$04.00+0

Identification of *Cryptosporidium felis* in a Cow by Morphologic and Molecular Methods

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Received 17 November 1998/Accepted 7 January 1999

Apicomplexan *Cryptosporidium* parasites infect a wide range of vertebrate hosts. While some species are limited to a single host group, such as *Cryptosporidium baileyi*, which infects chickens, other species of this genus, such as *C. parvum*, infect a wide range of mammalian species from mice to humans. During an investigation of *Cryptosporidium* infection in cattle on a farm in northern Poland, we identified an infection caused by *C. felis*, in addition to known infections with *C. muris* and *C. parvum*. This new infection was identified based on the size of the oocysts (mean size, 4.3 ± 0.4 μm; range, 3.5 to 5.0 μm), as well as by analysis of the molecular sequence of the variable region of the small-subunit rRNA. This finding demonstrates the complex host specificity and circulation in the environment of *Cryptosporidium* species.

Cryptosporidium is a coccidian genus belonging to the phylum Apicomplexa. The first species of this genus, which was found affecting the gastric glands of mice, was described by Tyzzer in 1907 (31a) and was named *Cryptosporidium muris*. Two years later, the same author described a similar organism in mice and named it *C. parvum*. This parasite had smaller oocysts than *C. muris* and was found infecting the small intestine. Infections caused by *C. parvum* in other hosts were first reported in 1971 in association with bovine diarrhea in young calves, sometimes accompanied by lethargy, decreased feeding, fever, dehydration, and/or poor condition. Conversely, an abomasum-infecting species resembling *C. muris* was reported in chronically infected adult cattle. The animals infected with this species had no diarrhea, although they excreted oocysts for several months. Milk production and usual weight gains were significantly reduced in *Cryptosporidium*-infected cattle. Natural infections caused by both species are highly prevalent in cattle, wild mice, and rats worldwide. Species identification is achieved by evaluation of morphometric characteristics of the excreted oocysts. The measurement ranges are 4.5 to 5.4 by 4.2 to 5.0 μm (average, 5.0 by 4.5 μm) and 6.6 to 7.9 by 5.3 to 6.5 (average, 7.4 by 5.6 μm) for *C. parvum* and *C. muris*, respectively. Nevertheless, these values may vary, depending on how the oocysts have been processed and whether or not they are fixed (11).

Early transmission experiments showed a lack of *Cryptosporidium* host species specificity within mammalian groups (26). However, the theory of a monotypic, single-species *Cryptosporidium* genus was misleading, having been proposed only because mammal isolates were transmitted to mammals (26). Numerous experimental cross-transmission studies have established that other *Cryptosporidium* species are specific to reptiles, avians, and mammals, and some species, such as *C. wrairi*, are even more specific, infecting only guinea pigs (13). Several *Cryptosporidium* species were named after the host of origin of the oocysts; however, subsequent cross-transmission experiments have invalidated many of these species (11). Currently, eight valid *Cryptosporidium* species are listed that infect all vertebrate groups: *C. nasorum* (fish), *C. serpentis* (reptiles), *C. baileyi* and *C. meleagridis* (birds), and *C. felis*, *C. wrairi*, *C. muris*, and *C. parvum* (mammals) (11). Mammalian cryptosporidiosis has been reported in at least 79 species, including humans (11). Apart from cryptosporidial infections in guinea pigs (*C. wrairi*) and cats (*C. felis*), the main species identified in mammals presents morphologic characteristics similar to those of *C. parvum* (26).

The lack of feasible in vitro cultivation systems for this parasite has been an obstacle to detailed biologic, immunologic, and molecular studies of different isolates resembling *C. parvum*. Recently, consistent results of *C. parvum* genotyping isolated from cattle and infected humans demonstrated the existence of two distinct genotypes. They are the human genotype, or genotype 1, found so far only in humans, and the bovine genotype, also known as genotype 2 or the cattle genotype, found either in humans or in livestock (21, 28, 31, 33). On the other hand, phylogenetic analysis of some *C. parvum*-like isolates from other hosts clustered them in distinct groups, indicating that they may actually be different *Cryptosporidium* species (21, 22, 29). Cryptosporidiosis in humans is attributed to *C. parvum* isolates of human or cattle origin (11, 26). However, a few reports have shown that other animals and other *Cryptosporidium* species may be associated with the disease in humans (9, 10, 17, 19). This hypothesis was not fully confirmed because no robust typing methodology was yet available.

Herein we report the characterization of a *C. felis*-like isolate obtained from a cow bred on the Bystra farm in the Gdańsk district of Poland by both molecular typing and morphometric evaluation of the oocysts. This isolate is identical to

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the isolate recently reported in cats (29) and in persons with AIDS (28a).

MATERIALS AND METHODS

Fecal specimens. *Cryptosporidium* infections were initially diagnosed by acid-fast staining after formol-ethyl acetate concentration (4) of fresh stools and stool smears. Fecal specimens from 342 animals were collected during an investigation of the prevalence of *Cryptosporidium* sp. infections at the Bystra farm (Gdańsk district of Poland). Microscopic investigations showed the presence of oocysts in 146 cows (42.7%). As preliminary investigations indicated that two sizes of oocysts were seen in the analyzed samples, specimens from six animals, three with large oocysts (PM348, PM586, and PM829) and three with small oocysts (PM681, PM741, and PM751), were selected for further investigations and preserved by 1:1 dilution with 5% (wt/vol) potassium dichromate (2). Preserved oocyst isolates were stored at 4°C for up to 10 months before DNA extraction.

Morphologic evaluation of oocysts. Slides with oocysts were initially examined with an Olympus microscope using a 40× objective. While in a 2.5% aqueous solution of potassium dichromate, the oocysts were identified and their diameter was measured ($n = 30$ of each isolate) using a 100× oil immersion objective.

Statistical analysis was carried out with Statistix 4.1 (Analytical Software, St. Paul, Minn.). The variables were examined by the Runs test to determine conformity to a normal distribution. Parametric tests included analysis of variance and a two-sample *t* test. Mean values and standard deviations were determined. Statistical significance was considered to be $P \leq 0.05$.

DNA extraction. An aliquot of about 300 μl of each stool specimen was suspended in 1 ml of phosphate-buffered saline (PBS; 0.01 M, pH 7.2) containing 0.01 M EDTA (PBS-EDTA), and the suspension was centrifuged at 14,000 × *g* and 4°C for 5 min. The pellet from this centrifugation was washed two more times under the conditions described above. The pellet was resuspended in 300 μl of PBS-EDTA and used for DNA extraction. DNA extraction was performed by using the FastPrep disrupter and the FastDNA kit (Bio 101, Inc., Vista, Calif.) by the method of da Silva et al. (8).

PCR amplification. *Cryptosporidium* genus-specific primers (CPBDIAGF and CPBDIAGR) were used for amplification of the variable region of the region coding for *Cryptosporidium* small-subunit (SSU) rRNA (15, 16). These primers bind to all known *Cryptosporidium* SSU rRNA sequences. For *C. parvum*, the diagnostic fragment amplified with these primers is 435 bp long from position 601 to position 1035 of the *C. parvum* SSU rRNA sequence (GenBank accession no. L16996). The conditions for PCR were 95°C for 15 min; 45 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 1.5 min; followed by extension at 72°C for 9 min and finished with a hold step at 4°C.

PCR products were analyzed by electrophoresis on 2% SeaKem GTG agarose (catalog no. 50074; FMC Bioproducts, Rockland, Maine), stained with ethidium bromide, and visualized on a UV transilluminator.

DNA sequencing and analysis. Amplification products were purified by using the Wizard PCR Preps kit (catalog no. A7170; Promega, Madison, Wis.). Sequencing reactions were performed by using the Perkin-Elmer Big Dye Kit (catalog no. 4303149; PE Biosystems, Foster City, Calif.) and analyzed on a Perkin-Elmer ABI 377 automatic DNA sequencer. Sequences were assembled by using the program SeqMan II (DNASTAR Inc., Madison, Wis.). Sequences were aligned with the program MACAW (30) and analyzed by using programs from the PHYLIP phylogeny inference program package (12).

Nucleotide sequence accession number. The sequence of the SSU rRNA diagnostic fragment of specimen PM741 was submitted to GenBank (accession no. AF087577).

RESULTS

Morphometric analysis of oocysts. As determined by analysis of variance, the differences in diameter among the six oocyst isolates were significant ($F = 44.3$, $P < 0.01$) (Table 1). Two-sample *t*-test analysis divided the six oocyst isolates into two groups (group I, PM348, PM586, and PM829; group II, PM681, PM741, and PM751), confirming the difference seen during the preliminary investigation.

PCR analysis. Agarose gel visualization of PCR products revealed two different diagnostic fragment sizes among the samples tested. Whereas the diagnostic fragments amplified from samples PM348, PM586, and PM829 matched the size of the fragment amplified from the *C. muris* cloned SSU rRNA region (431 bp; GenBank accession no. L19069, Fig. 1, lane 2 [the results shown are for specimen PM348]), the diagnostic fragments amplified from samples PM681, PM741, and PM751 were visibly larger, about 450 bp (Fig. 1, lane 1 [the results shown are for specimen PM741]).

TABLE 1. Sizes of *Cryptosporidium* oocysts recovered from the feces of six cows[a]

Bovine fecal sample	Mean oocyst diam (μm) ± SD (range)
PM348	5.8 ± 0.8 (4.0–7.4) A
PM586	6.1 ± 0.8 (4.6–7.8) A
PM681	4.5 ± 0.4 (3.9–5.3) B
PM741	4.6 ± 0.5 (3.8–5.6) B
PM751	4.3 ± 0.4 (3.5–5.0) B
PM829	6.5 ± 1.0 (4.9–7.9) A

[a] Thirty oocysts from each cow were measured. A-versus-B differences are statistically significant by the two-sample *t* test ($P < 0.01$). A-versus-A and B-versus-B differences are not statistically significant by the two-sample *t* test ($P > 0.05$).

DNA sequencing analysis of amplified products. Specimens PM348 and PM741 generated adequate amounts of the PCR products and were selected for sequencing. Sequence analysis revealed two types of sequences. The DNA sequence of the *Cryptosporidium* diagnostic fragment from sample PM348 was 431 bases long and was identical to the region from position 602 to position 1032 of the *C. muris* SSU rRNA (GenBank accession no. L19069). The length of the diagnostic fragment generated from sample PM741 was 455 bp. GenBank database searches and phylogenetic analysis revealed that while this sequence was not identical to any known sequences in the GenBank database, it clustered within other representative SSU rRNA sequences from the genus *Cryptosporidium* (results not shown). Moreover, it was significantly different from partial SSU rRNA sequences recently reported for several genotypes of *C. parvum* (6, 21, 23). The sequence of isolate PM741 had only five differences in a 100-bp overlap with the feline *C. parvum* genotype (or *C. felis*) recently published (29). These differences may have been the result of sequencing artifacts not corrected in the report of Sargent et al. (29). A *C. felis* sequence recently submitted to GenBank by the same authors (GenBank accession no. AF097430) was identical to that of

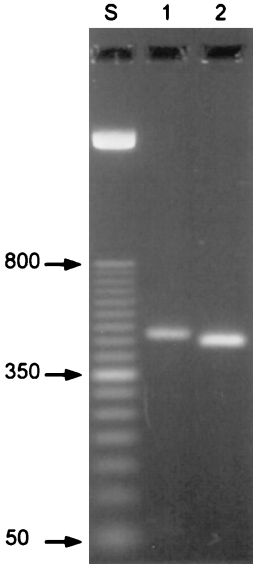

FIG. 1. PCR detection of *Cryptosporidium*-specific diagnostic fragments. Lanes: 1, cow isolate PM741; 2, cow isolate PM348; S, 50-bp ladder used as a DNA fragment size standard. The values beside the gel are DNA fragment sizes in base pairs. The expected *C. muris*-specific diagnostic band size is 431 bp, and the *C. felis*-specific fragment is 455 bp.

isolate PM741 in the 100-bp overlap. In addition, the sequence of the SSU rRNA diagnostic fragment of specimen PM741 was found to be identical to the sequence of several *Cryptosporidium* isolates from human patients (28a).

DISCUSSION

C. muris and *C. parvum* commonly cause bovine cryptosporidiosis. In our study of the prevalence of *Cryptosporidium* infections on the Bystra farm in northern Poland, *Cryptosporidium* oocysts were found in 42.7% of the 342 animals examined. Examination by microscopy revealed the presence of small and large oocysts in 82.5 and 17.5%, respectively, of the animals passing *Cryptosporidium* oocysts. The analysis of these samples also demonstrated the presence of both oocyst sizes in 5.5% of the infected animals (25a).

To date, all ruminant (bovine, caprine, ovine, and cervine) *Cryptosporidium* isolates that had small oocysts were named *C. parvum* by default. These oocyst isolates were also identified in human patients and are commonly referred to as *C. parvum* genotype 2, bovine, or zoonotic genotype (5, 21, 28, 31, 33). On the other hand, the human isolate (anthroponotic or genotype 1) of *C. parvum* has never been reported in naturally infected animals. Additional *C. parvum*-like isolates found in koalas, mice, pigs, and cats were demonstrated to be distinct from genotypes 1 and 2 on the basis of their SSU rRNA sequence (21, 23, 29).

Among these new isolates, only a *Cryptosporidium* sp. found in cats was formerly proposed to be a distinct species, *C. felis* (14). Epidemiologic surveys of felines conducted in Australia, Japan, Germany, Italy, the United States, and the United Kingdom identified *Cryptosporidium* oocysts in 0.75 to 12.2% of the cats (11, 18, 29) and established the presence of specific antibodies in 15.3 to 87% of the animals (18, 24, 32). Morphometric analysis of some feline oocysts revealed similar oocyst measurements: 4.3 by 4.7 μm (1), 4.5 ± 0.22 μm (3), 3.8 to 4.8 by 4.6 to 4.9 μm (25), and 4.0 by 4.6 μm (29). The sizes of the oocysts found in cows PM681, PM741, and PM751 are similar to these (4.3 to 4.5 ± 0.5 μm), being smaller than those established for *C. parvum* oocysts.

The sequence analysis of the SSU rRNA diagnostic fragment obtained for sample PM348 showed that it was 100% identical to the same region of *C. muris*, providing the definitive identification of this isolate as *C. muris*, as indicated by the size of the oocysts. Conversely, the sequence in oocysts isolated from animal PM741 differed from sequences previously obtained from *Cryptosporidium* oocysts isolated from cattle and analyzed by others (6, 21, 23) but was identical to the sequence reportedly found in a *C. parvum*-like isolate from domestic cats (29) and in human immunodeficiency virus-infected patients (28a). The five discrepancies in a 100-bp overlap between our sequence and the sequence published by Sargent et al. (29) were clarified as sequencing artifacts, as the sequence submitted by them to GenBank (accession no. AF097430) was identical to our sequence. The consistent finding of a characteristic molecular sequence among *C. felis* isolates from different hosts, e.g., cows, cats, and humans, collected in different geographic regions, i.e., Poland, Australia, and the United States, strongly indicates that all of these isolates represent a unique and conserved species of *Cryptosporidium*.

Experimental infection with feline *Cryptosporidium* isolates failed to establish infection in mice, rats, guinea pigs, and dogs (20). Infection with cat oocyst isolates succeeded only in lambs and other cats (3, 25). Furthermore, cats were experimentally infected by *Cryptosporidium* oocyst isolates obtained from human and bovine hosts (7, 27). These data, together with the reports identifying cats as a reservoir of *Cryptosporidium* and a source for human infection (10, 17, 19), suggest that the host specificity of some of the *Cryptosporidium* species infecting mammals may be less restricted than currently thought. Nevertheless, the final proof of the identity of the *Cryptosporidium* isolate, or species, used in transmission studies should depend on a more stringent tool than morphometric analysis of the oocysts, e.g., molecular typing of the *Cryptosporidium*-specific diagnostic fragment of the SSU rRNA. In addition, a combination of data from different molecular markers will also be useful for typing of *Cryptosporidium* isolates obtained from various hosts, immunocompetent and immunodeficient individuals, and environmental sources. Biologic studies (e.g., experimental infection, culturing, and drug testing), as well as biochemical and immunologic analysis of newly identified genotypes of *Cryptosporidium* spp., will be of value for accurate classification of *Cryptosporidium* spp. and for understanding the epidemiology of cryptosporidial infections in humans.

ACKNOWLEDGMENTS

This work was supported by grant MZ/NIH-96-288 from U.S.-Poland Maria Skłodowska-Curie Joint Fund II.

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