

## CRYPTOSPORIDIUM CANIS N. SP. FROM DOMESTIC DOGS

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**ABSTRACT:** Oocysts of *Cryptosporidium*, from the feces of a naturally infected dog and from an HIV-infected human, were identified as the previously reported canine genotype of *Cryptosporidium parvum*, hereafter referred to as *Cryptosporidium canis* n. sp. Also among the oocysts from the dog, a trace amount of *C. parvum* bovine genotype was detected. *Cryptosporidium canis* oocysts from both the dog and human were infectious for calves. Oocysts excreted by calf 1 (dog source) were approximately 90% *C. canis* and 10% *C. parvum*, whereas those excreted by calf 3 (human source) were 100% *C. canis*. Oocysts from calf 1 infected calf 2 resulting in excretion by calf 2 of oocysts ~90% *C. parvum* and 10% *C. canis*. Oocysts of *C. canis* were not infectious for BALB/c neonatal mice or immunosuppressed C57 juvenile mice, although all control mice became infected with the *C. parvum* Beltsville isolate. Oocysts of *C. canis* from calf 1 and the human were structurally indistinguishable from oocysts of the *C. parvum* Beltsville isolate (bovine). However, *C. canis* oocysts differed markedly at the molecular level from all known species of *Cryptosporidium* based on sequence data for the 18S rDNA and the HSP 70 gene. The differences in genetics and host specificity clearly differentiate *C. canis* as a new species.

The first report referring to cryptosporidial infection in dogs indicated that antibody to *Cryptosporidium* was found in sera from 16 of 20 dogs (Tzipori and Campbell, 1981). Two years later, the first clinical case of canine cryptosporidiosis, along with the first description of life cycle stages, was reported (Wilson and Holscher, 1983). Subsequently, reports have followed 2 general themes based on finding oocysts in feces: (1) case reports of chronic clinical illness in which dogs appeared immunosuppressed because of a concurrent illness or toxicity (Sisk et al., 1984; Dominguez and Almarza, 1988; Denholm et al., 2001) and (2) surveys conducted to determine the prevalence of infected dogs within larger populations. In the former group, for example, naturally infected pups immunosuppressed by distemper virus infection developed persistent diarrhea (Fukushima and Helman, 1984; Turnwald et al., 1988) and a 5-yr-old, naturally infected, male Pointer with chronic neutropenia developed persistent diarrhea (Greene et al., 1990). Overall in the latter group, both the prevalence and number of oocysts observed in each fecal specimen (when reported) from dogs from Scotland, France, Finland, Egypt, various locations in Australia, and the United States, Japan, and Korea have appeared rather low (Table I). All of these prevalence studies have relied on morphometric identification of the parasite and have not attempted to identify species by other methods. Therefore, no information is available to either confirm the identity of the species infecting dogs as *Cryptosporidium parvum* or to identify a genotype within that species.

Within *C. parvum*, several unique genotypes have now been identified in association with specific hosts such as human, mouse, pig, marsupial, dog, and ferret based on gene sequence data (Morgan et al., 1999; Xiao et al., 1999). The oocyst stage of each genotype is indistinguishable from that of other genotypes, and limitations based on biological features have restricted our ability to clearly identify them as species.

The present study was undertaken to examine oocysts from a dog and an HIV-infected human that matched a previously described genotype of *C. parvum* associated with dogs and to

determine if these oocysts differed enough from known species of *Cryptosporidium* to be considered a new species. Differences to be examined would include oocyst morphology, host specificity, and gene sequencing.

### MATERIALS AND METHODS

#### Source of oocysts

The oocysts obtained from the feces of a 25-kg, 16-mo-old, female, mixed-breed dog purchased from a licensed animal dealer were used to determine molecular characteristics as well as potential for transmission to mammalian hosts. Initially, feces from this dog were found negative for *Cryptosporidium*. One day after receiving the last of 3 intramuscular injections of methyl prednisolone (200, 400, and 200 mg over 3 wk), the dog excreted *Cryptosporidium* oocysts for only 2 days. Oocysts from a calf (calf 1) experimentally infected with the oocysts from this dog were examined for morphometric, molecular, and transmission characteristics. Additional oocysts from the feces of an adult male citizen of Peru with HIV infection were shipped to the CDC, where a portion were examined for molecular characteristics and the remainder were shipped to the U.S. Department of Agriculture (USDA), where they were measured and tested for animal infectivity.

#### Oocyst preparation

Oocysts, initially from the dog and the human, and those later obtained from mice and calves, as well as the bovine *C. parvum* Beltsville isolate, were cleaned of large fecal debris by washing through a graded series of sieves down to a pore size of 45  $\mu\text{m}$ . Smaller debris was removed by density gradient centrifugation over cesium chloride as previously described (Kilani and Sekla, 1987). Residual cesium chloride was removed by 3 cycles of centrifuging at 1,000 g for 10 min, aspirating the supernatant, and resuspending pelleted oocysts in distilled water. Cleaned oocysts were stained with *Cryptosporidium/Giardia* test reagents (Mer/Fluor<sup>®</sup>, Meridian Diagnostics, Cincinnati, Ohio) and observed by immunofluorescence (IF), interference contrast (DIC), and brightfield (BF) microscopy. Oocysts were photographed using DIC microscopy and phototypes were deposited in the U.S. National Parasite Collection, Beltsville, Maryland.

#### Host specificity

All animals received  $1 \times 10^6$  oocysts less than 2-mo-old. Aqueous suspensions of oocysts were administered to calves via nipples bottles (Table II). Calf 1 received oocysts from the dog. Calf 2 received oocysts excreted by calf 1. Calf 3 received oocysts from the human. Calf 4 received *C. parvum* oocysts of the Beltsville isolate from stock cultures maintained in the USDA laboratory. Oocysts from the dog were also administered orally by intubation with a 26-gauge gavage needle into the stomachs of 3- to 5-day-old BALB/c and 8-wk-old C57bl6/N (C57) mice. Within each group, 4 to 10 test mice received oocysts from the dog at the same time that 4 to 8 control mice received oocysts of the bovine *C. parvum* Beltsville isolate. One group of C57 mice was im-

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TABLE I. Prevalence of *Cryptosporidium* oocysts reported in domestic dogs.

| Location                         | No. positive/No. sampled<br>(% positive) | Reference                       |
|----------------------------------|--|---------------------------------|
| Finland                          | 0/57 (0)                                 | Pohjola, 1984                   |
| Scotland                         | 0/101 (0)                                | Simpson et al., 1988            |
| Japan                            | 3/213 (1.4)                              | Uga et al., 1989                |
| San Bernadino, California        | 4/200 (2)                                | El Ahraf et al., 1991           |
| Western Scotland (public parks)  | 1/100 (1)                                | Grimason et al., 1993           |
| Georgia, U.S.A.                  | 5/49 (10.2)                              | Jafri et al., 1993              |
| Melbourne and Geelong, Australia |  | Johnston and Gasser, 1993       |
| Stray dogs                       | 29/190 (15.3)                            |                                 |
| Kennels                          | 3/44 (6.8)                               |                                 |
| Parks                            | 21/107 (19.6)                            |                                 |
| Veterinary clinics               | 1/152 (0.7)                              |                                 |
| Hobart, Australia                |  | Milstein and Goldsmid, 1995     |
| Urban dogs                       | 1/55 (1.8)                               |                                 |
| Parks and beaches                | 13/142 (9.2)                             |                                 |
| France                           | 13/29 (44.8)                             | Chermette and Blondel, 1989     |
| Egypt                            | 3/25 (12)                                | El Hohary and Abdel-Latif, 1998 |
| Kentucky, U.S.A.                 | 17/100 (17)                              | Juett et al., 1996              |
| Korea                            | 25/257 (9.7)                             | Kim et al., 1998                |
| Perth, Australia                 | 0/421 (0)                                | Bugg et al., 1999               |

munosuppressed by the addition of dexamethasone to their standard drinking water at the rate of 0.01 mg/ml beginning 2 days before inoculation and lasting for 7 consecutive days (Table II).

Feces were collected from each calf and specific groups of mice daily from 4 to 10 days after inoculation and examined for the presence of oocysts by IF microscopy (Table II). Tissue segments of duodenum, jejunum, and ileum were taken for histology from mice in groups 2 and 3 (Table II) 6 days after inoculation; the same tissues were taken from group 1 (Table II) 4 days after inoculation. All tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, stained with hematoxylin-eosin, and examined by BF microscopy for stages of *Cryptosporidium*.

#### Morphometric analysis

Cleaned oocysts from calf 1 were measured with the aid of a micrometer in the eyepiece of a Zeiss Axioskop microscope using a  $\times 100$  PlanNeofluor objective. Fifty oocysts were measured with BF optics and 50 with DIC optics at the USDA laboratory. Oocysts from the human source were measured by the same person using the same mi-

croscope equipment; 50 oocysts were measured with BF optics and 50 with DIC optics. Likewise, oocysts of *C. parvum* Beltsville isolate, obtained from calf 4, were measured (Table III).

#### Genetic analysis

Oocysts cleaned of fecal debris from the dog, the C57 mice, and calves 1 and 2, as well as formalin-fixed, paraffin-embedded ilea from BALB/c mice (group 2, Table II) were shipped to the Centers for Disease Control and Prevention laboratory for molecular examination. Oocysts from the HIV-infected human were also examined in this laboratory. DNA extracted from all specimens was subjected to amplification by polymerase chain reaction (PCR) and then sequenced and compared with sequences from other human and animal isolates of cryptosporidial species. Primary characterization of the cryptosporidial parasites from the dog and calves was conducted at the small-subunit (SSU) rRNA gene locus. An 831-bp segment of the SSU rRNA gene was amplified by nested PCR. Primers and amplification conditions used in this study were previously described (Xiao, Escalante, Yang et al., 1999; Xiao, Morgan, Limor et al., 1999), except that the reverse

TABLE II. Identification of *Cryptosporidium* in feces or tissues of laboratory mice and calves orally inoculated with *Cryptosporidium* oocysts from canine, bovine, and human sources. All histology specimens were acquired 6 days after inoculation, except group 1, which was acquired at 4 days.

| Group | Recipients  | Age    | Source of inoculum* |        |           |        |           |             |
|-------|-------------|--------|---------------------|--------|-----------|--------|-----------|-------------|
|       |             |        | Dog feces           | Calf 1 |           | Calf 4 |           | Human feces |
|       |             |        |                     | Feces  | Histology | Feces  | Histology |             |
| 1     | BALB/c mice | <1 wk  |                     | ND     | 0/4       | ND     | 4/4       |             |
| 2     | BALB/c mice | <1 wk  |                     | ND     | 8/8       | ND     | 8/8       |             |
| 3     | C57 mice†   | 8 wk   |                     | 8/8 B  | 2/2       | 8/8 B  | ND        |             |
| 4     | C57 mice‡   | 8 wk   |                     | 0/8    | ND        | 0/8    | ND        |             |
| 5     | Calf 1      | 2 days | 1/1 BC              |        |           |        |           |             |
| 6     | Calf 2      | 2 days |                     | 1/1 CB |           |        |           |             |
| 7     | Calf 3      | 2 days |                     |        |           |        |           | 1/1 C       |

\* Number of positive specimens/number of animals examined. ND, not done; B, genotype *C. parvum* bovine; C, genotype *C. canis*.

† Dexamethasone treatment.

‡ No dexamethasone treatment.

TABLE III. Morphometric analysis of oocysts.

| Source   | Optics | n  | Mean measurements           |                            |       | Range<br>( $\mu\text{m}$ ) |
|----------|--------|----|-----------------------------|----------------------------|-------|----------------------------|
|          |        |    | Length<br>( $\mu\text{m}$ ) | Width<br>( $\mu\text{m}$ ) | Ratio |                            |
| Calf 1*† | BF     | 50 | 4.62                        | 4.44                       | 1.04  | 3.68–5.88 × 3.68–5.88      |
| Calf 1*† | DIC    | 50 | 4.85                        | 4.65                       | 1.04  | 4.41–5.15 × 3.68–5.15      |
| Human*   | BF     | 50 | 5.02                        | 4.75                       | 1.06  | 3.68–5.88 × 3.68–5.15      |
| Human*   | DIC    | 50 | 5.32                        | 5.00                       | 1.06  | 4.78–5.88 × 4.41–5.88      |
| Calf 4‡  | BF     | 50 | 5.00                        | 4.70                       | 1.06  | 4.70–6.00 × 4.41–5.00      |
| Calf 4‡  | DIC    | 50 | 5.37                        | 5.10                       | 1.05  | 4.76–5.95 × 4.76–5.95      |

\* *Cryptosporidium canis*.

† These measurements are a composite of predominantly *C. canis* and some *C. parvum* oocysts; actual numbers of each could not be determined.

‡ *Cryptosporidium parvum* Beltsville isolate.

primer used in the primary PCR was 5'-CCCATTTCCTTCGAAACAGGA-3'. For genotyping analysis, restriction fragment length polymorphism (RFLP) was assessed by the digestion of the secondary PCR product with *SspI* and *VspI* (Fig. 4). The secondary PCR product was further sequenced in both directions on an ABI377 autosequencer (Applied Biosystems, Foster City, California). In addition, a 1,920-bp fragment of the gene coding for the 70-kDa heat shock protein (HSP 70) was further sequenced from PCR-amplified products as previously described (Sulaiman et al., 2000). The SSU rRNA and HSP 70 sequences were then compared with sequences previously obtained from the dog genotype and other cryptosporidial parasites (Xiao, Escalante, Yang et al., 1999; Xiao, Morgan, Limor et al., 1999; Sulaiman et al., 2000). Genetic distances among different cryptosporidial parasites were calculated using the Kimura 2-parameter model (Xiao, Escalante, Yang et al., 1999).

## DESCRIPTION

### *Cryptosporidium canis* n. sp.

(Figs. 1–3)

#### Taxonomic summary

**Diagnosis:** Two-hundred oocysts had an average length, width, and length/width ratio (l/w) of 4.95, 4.71, and 1.05  $\mu\text{m}$ , respectively. The size range for oocysts was 3.68–5.88 by 3.68–5.88  $\mu\text{m}$ , and the l/w range was 1.04–1.06. Each oocyst was colorless, nearly spherical, and contained 4 sporozoites and a few residual granules. Sporozoites were not easily seen within oocysts.

**Type definitive host:** Dog (*Canis familiaris*).

**Other definitive hosts:** Human (*Homo sapiens*).

**Type location:** Maryland, United States.

**Additional locations:** Ohio and Georgia, United States; Australia, Peru.

**Experimental definitive hosts:** Bovine (*Bos taurus*).

**Specimens deposited:** Phototypes were deposited in the U.S. National Parasite Collection, Beltsville, Maryland, as USNPC No. 90587 on 1 August 2000.

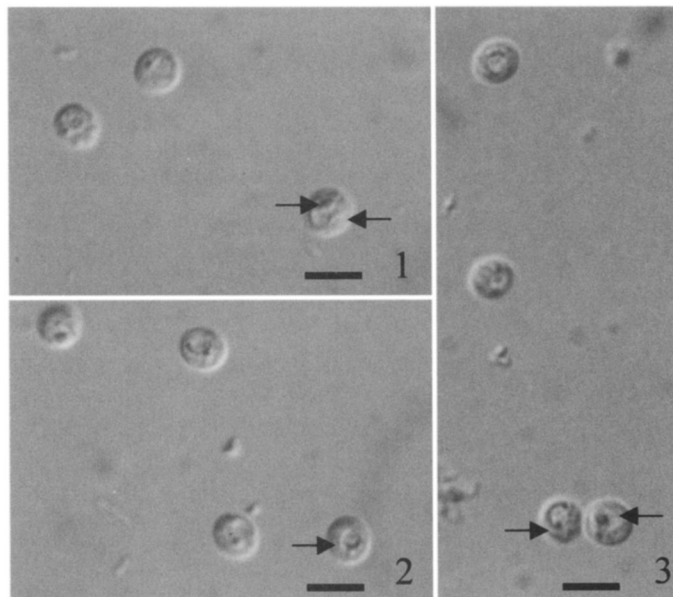
**Etymology:** *Cryptosporidium canis* is named for the domestic dog in the genus *Canis*, because the type specimens were based on genetic sequences obtained from oocysts that have ultimately and repeatedly been isolated from *Canis familiaris*, the type host.

#### Remarks

Oocysts of *C. canis* are morphologically indistinguishable from, and possess surface antigens in common with, those of the human and bovine genotypes of *C. parvum*. Measurements for length, width, and the l/w ratios are presented in Table III. Unlike the bovine genotype of *C. parvum*, *C. canis* is not infectious for mice, even when they have been immunosuppressed. Unlike the human genotype of *C. parvum*, *C. canis* is infectious for cattle. *Cryptosporidium canis* differs markedly at the molecular level from all known species of *Cryptosporidium* based on sequence data for the 18S rDNA and the HSP 70 gene.

Oocysts from feces of the dog consisted of a mixture of *C. parvum*

bovine genotype and *C. canis*. They were infectious for BALB/c neonatal mice, immunosuppressed C57 juvenile mice, and a newborn Holstein (calf 1), as determined by histologic or fecal examination (Table II). Similar levels of tissue infection or oocyst excretion were observed in mice infected with oocysts from the dog and in control mice infected with oocysts from calf 1, with the following exceptions. When BALB/c mouse tissues were taken 4 days after inoculation, developmental stages were not found in mice that received oocysts from the dog, but stages were observed in mice that received oocysts from calf 1. When C57 mice were not immunosuppressed, oocysts were not detected in the feces of mice that received oocysts from either the dog or calf. A persistent difference for all C57 mice examined for oocyst excretion as an indicator of infectivity was that the prepatent period for mice that received oocysts from the dog was 2 or 3 days longer than the prepatent period for those that received oocysts from the calf. Consequently, examination of histologic sections from BALB/c mice 4 days after inoculation revealed no developmental stages in mice that received oocysts from the dog, but numerous stages in mice that received oocysts from the calf. Molecular analysis of oocysts collected from feces of BALB/c and C57 mice that received



FIGURES 1–3. Photomicrographs of *C. canis* oocysts from dog feces taken with the aid of differential interference contrast microscopy (1 and 2) and phase-contrast microscopy (3). Magnification  $\times 1,500$ . Bar = 6  $\mu\text{m}$ . 1. Oocyst with 2 sporozoites visible (arrows). 2. Three oocysts, each with a distinct, prominent, eccentric granule and 1 oocyst (arrow) with a central globule. 3. Two oocysts each with a central, light-density area containing an eccentric dark granule (arrows).

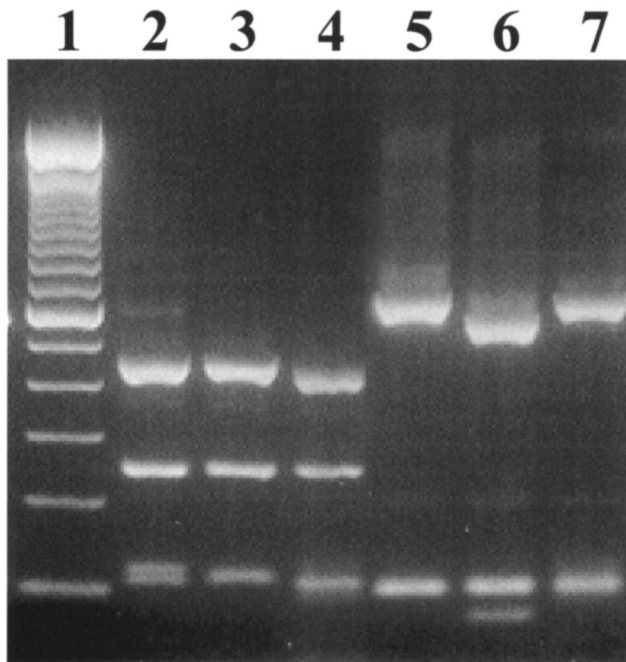


FIGURE 4. PCR-RFLP analysis of an 831-bp region of the SSU rRNA gene of the dog *Cryptosporidium* isolate. Lane 1: 100-bp molecular ladders; lanes 2, 5: *C. parvum* bovine genotype; lanes 3, 6: *C. parvum* human genotype; lanes 4, 7: *Cryptosporidium* dog genotype. Lanes 2–4: *SspI* digestion; lanes 5–7: *VspI* digestion.

oocysts from the dog indicated that the mice were excreting only *C. parvum* oocysts of the bovine genotype (Table II). Furthermore, calf 1, which received oocysts from the dog, excreted  $>10^7$  oocysts over a period of 10 consecutive days, beginning 5 days after inoculation. These oocysts were approximately 90% *C. canis* and 10% *C. parvum* bovine genotype based on the density of PCR-RFLP bands. Calf 2, which received oocysts from calf 1, excreted approximately 90% *C. parvum* bovine genotype and 10% *C. canis* oocysts based on the density of PCR-RFLP bands. Calf 3, which received oocysts from a human source, excreted only *C. canis* oocysts based on PCR-RFLP analysis.

PCR-RFLP analysis of the SSU rRNA gene showed that *C. canis* oocysts from the dog had *SspI* and *VspI* restriction patterns different from *C. parvum*, but identical to the genotype previously described from dog-derived oocysts (Xiao, Escalante, Yang et al., 1999; Morgan et al., 1999; Morgan, Xiao, Monis et al., 2000). Compared with the bovine and human genotypes of *C. parvum*, *C. canis* had a smaller upper *SspI* digestion band (Fig. 4). This pattern was also seen in DNA isolated from *C. canis* oocysts recovered from calves 1 and 2. DNA sequence analysis of the SSU rRNA PCR products from *C. canis* oocysts from the dog and from calves 1 and 2 revealed that this parasite was identical to the dog genotype of *Cryptosporidium* previously found in the United States and Australia (Xiao, Escalante, Yang et al., 1999; Morgan et al., 1999; Morgan, Xiao, Monis et al., 2000). In the 831-bp region examined, compared to the bovine genotype of *C. parvum*, *C. canis* had 20 bp substitutions, 1 bp insertion, and 6 bp deletions. Likewise, *C. canis* also had 21 bp substitutions, 1 bp insertion, and 9 bp deletions compared with the human genotype of *C. parvum*. PCR-RFLP and sequence analysis of the SSU rRNA also indicated minute traces of the bovine genotype in oocysts from the dog and from calf 1. DNA extracted from oocysts recovered from mice was subjected to PCR-RFLP analysis of

the SSU rRNA gene and were found to be identical to the *C. parvum* bovine genotype with no trace of *C. canis* (Table II).

The 1,920-bp fragment of the HSP 70 gene from oocysts from the dog was found to be identical to previously analyzed oocysts isolated from dogs (Morgan, Xiao, Monis et al., 2000; Sulaiman et al., 2000). *Cryptosporidium canis* had 261 bp changes compared with the *C. parvum* bovine genotype and 265 bp changes compared with the *C. parvum* human genotype. The nucleotide differences involved 7 amino acid changes representing 2.5% of total mutations. Differences among the bovine and human genotypes of *C. parvum*, *C. felis*, *Cryptosporidium* sp. from a bear, and *C. canis* in the SSU rRNA and HSP 70 genes are shown in Figure 5. Sequences for *C. canis* submitted to GenBank have accession numbers AF112576 and AF221529.

The genetic distances and the relationships among different *Cryptosporidium* parasites, based on the SSU rRNA and the HSP 70 sequence data, were determined using the Kimura 2-parameter model (Xiao, Escalante, Yang et al., 1999; Xiao, Morgan, Limor et al., 1999; Sulaiman et al., 2000; Xiao, Limor et al., 2000) (Table IV). With the exception of *Cryptosporidium* sp. from a bear, the genetic differences between *C. canis* and other *Cryptosporidium* spp. were 3.29% or greater. This was greater than the genetic distance between *C. parvum* and *C. wrairi* (0.6%), between *C. parvum* and *C. meleagridis* (1.32%), or among *C. serpentis*, *C. muris*, and *C. andersoni* (0.60–2.32%). The genetic uniqueness of *C. canis* was more obvious in the HSP 70 gene, with a  $\geq 13.54\%$  difference between *C. canis* and other *Cryptosporidium* spp., which was much greater than the genetic distances among the aforementioned *Cryptosporidium* spp. (1.66–5.17%).

Phylogenetic analysis supported the validity of *C. canis* (Fig. 6). *Cryptosporidium canis* clustered with a *Cryptosporidium* sp. from a bear in neighbor-joining analysis of the SSU rRNA gene sequences. This cluster was separated from various *C. parvum* genotypes, *C. meleagridis*, and *C. wrairi* (Fig. 6A). Similarly, *C. canis* clustered with a clade containing the bear *Cryptosporidium* parasite and *C. felis* in the phylogenetic analysis of HSP 70 sequences and was separated from the major cluster containing most *C. parvum* genotypes, *C. wrairi*, and *C. meleagridis* (Fig. 6B).

## DISCUSSION

Unlike the bovine genotype of *C. parvum*, *C. canis* is not infectious for mice even when they have been immunosuppressed. Unlike the human genotype of *C. parvum*, *C. canis* is infectious for cattle. *Cryptosporidium canis* differs markedly at the molecular level from all known species of *Cryptosporidium*, based on sequence data for the 18S rDNA and the HSP 70 gene.

Oocysts of *C. canis* measured by the same person using the same microscope were 3.68–5.88 by 3.68–5.88  $\mu\text{m}$  with a mean size of 4.95 by 4.71  $\mu\text{m}$  ( $n = 200$ ) (Table III). These were similar in size to oocysts from other canines measured by Morgan, Xiao, Monis et al. (2000), which averaged 4.9 by 4.4  $\mu\text{m}$  ( $n = 20$ ). Oocysts in the present study also overlapped the size of *C. parvum* oocysts from bovine and human sources. In the present study, oocysts of *C. parvum* bovine genotype were 4.7–6.0 by 4.41–5.95  $\mu\text{m}$  with a mean size of 5.19 by 4.90  $\mu\text{m}$  compared with a previously reported range of 4.5–5.4 by 4.2–5.0  $\mu\text{m}$  and a mean size of 5.0 by 4.5  $\mu\text{m}$  (Upton and Current, 1985). Oocysts of a *C. parvum* human source were 3.8–6.0 by 3.0–5.3  $\mu\text{m}$  with a mean size of 5.0 by 4.5  $\mu\text{m}$  (Mercado and Santander, 1995). Furthermore, the morphometric feature, the mean shape index, also overlapped between species (1.04 for

FIGURE 5. Nucleotide sequence differences among human and bovine genotypes of *C. parvum*, *C. canis* (dog), *C. felis* (cat), and *Cryptosporidium* spp. in bear and pig in the diagnostic regions of the SSU rRNA (A) and HSP70 (B) genes. Dots denote nucleotide identity to the bovine genotype; dashes depict nucleotide deletion. (Based on GenBank AF09489, AF093490, AF112575, AF112576, AF115377, AF221528, AF221529, AF221533, AF221535, AF221538, AF247535, and AF247536.)



TABLE IV. Genetic distances (nucleotide changes per 100 bp calculated using Kimura 2-parameter model), recalculated from data by Xiao et al. (1996, 1999), Sulaiman et al. (2000), and Xiao, Limor et al. (2000), among *Cryptosporidium* spp. in the SSU rRNA and HSP 70 genes.

|                                 | <i>C. parvum</i> | <i>C. wrairi</i> | <i>C. malea-<br/>gridis</i> | <i>Cryptospo-<br/>ridium</i><br>sp. bear | <i>C. canis</i> | <i>C. felis</i> | <i>C. sauro-<br/>philum</i> | <i>C. baileyi</i> | <i>C. ander-<br/>soni</i> | <i>C. muris</i> |
|---------------------------------|------------------|------------------|-----------------------------|--|-----------------|-----------------|-----------------------------|-------------------|---------------------------|-----------------|
| SSU rRNA                        |                  |                  |                             |  |                 |                 |                             |                   |                           |                 |
| <i>C. wrairi</i>                | 0.60             |                  |                             |  |                 |                 |                             |                   |                           |                 |
| <i>C. maleagridis</i>           | 1.32             | 1.56             |                             |  |                 |                 |                             |                   |                           |                 |
| <i>Cryptosporidium</i> sp. bear | 2.67             | 2.79             | 2.79                        |  |                 |                 |                             |                   |                           |                 |
| <i>C. canis</i>                 | 3.29             | 3.41             | 3.29                        | 1.69                                     |                 |                 |                             |                   |                           |                 |
| <i>C. felis</i>                 | 4.04             | 4.03             | 4.41                        | 3.91                                     | 3.92            |                 |                             |                   |                           |                 |
| <i>C. saurophilum</i>           | 2.91             | 2.91             | 2.91                        | 3.29                                     | 3.79            | 4.03            |                             |                   |                           |                 |
| <i>C. baileyi</i>               | 4.70             | 4.95             | 4.83                        | 5.62                                     | 4.84            | 5.33            | 4.70                        |                   |                           |                 |
| <i>C. andersoni</i>             | 7.09             | 7.08             | 7.09                        | 7.64                                     | 7.38            | 8.14            | 7.07                        | 5.40              |                           |                 |
| <i>C. muris</i>                 | 7.22             | 7.20             | 7.22                        | 7.76                                     | 7.23            | 8.40            | 7.33                        | 5.66              | 0.60                      |                 |
| <i>C. serpentis</i>             | 6.94             | 6.93             | 7.21                        | 7.09                                     | 7.36            | 7.46            | 6.27                        | 5.27              | 1.95                      | 2.32            |
| HSP 70                          |                  |                  |                             |  |                 |                 |                             |                   |                           |                 |
| <i>C. wrairi</i>                | 1.82             |                  |                             |  |                 |                 |                             |                   |                           |                 |
| <i>C. maleagridis</i>           | 4.23             | 4.01             |                             |  |                 |                 |                             |                   |                           |                 |
| <i>Cryptosporidium</i> sp. bear | 11.23            | 10.80            | 11.19                       |  |                 |                 |                             |                   |                           |                 |
| <i>C. canis</i>                 | 15.54            | 17.26            | 16.14                       | 13.54                                    |                 |                 |                             |                   |                           |                 |
| <i>C. felis</i>                 | 17.53            | 18.17            | 18.58                       | 17.36                                    | 15.60           |                 |                             |                   |                           |                 |
| <i>C. saurophilum</i>           | 13.93            | 12.47            | 13.68                       | 16.49                                    | 20.78           | 19.43           |                             |                   |                           |                 |
| <i>C. baileyi</i>               | 15.62            | 15.88            | 17.16                       | 19.52                                    | 27.97           | 25.75           | 14.07                       |                   |                           |                 |
| <i>C. andersoni</i>             | 20.40            | 21.01            | 20.90                       | 23.19                                    | 28.83           | 26.89           | 19.56                       | 15.26             |                           |                 |
| <i>C. muris</i>                 | 18.71            | 19.36            | 19.73                       | 21.37                                    | 28.03           | 25.32           | 18.25                       | 14.73             | 1.66                      |                 |
| <i>C. serpentis</i>             | 21.44            | 20.59            | 21.18                       | 23.91                                    | 29.51           | 27.93           | 21.04                       | 15.72             | 5.17                      | 4.35            |

*C. canis* from a calf and 1.06 from a human vs. 1.05 and 1.06 for the bovine genotype of *C. parvum*). In addition, commercially available monoclonal antibody against *C. parvum* used for immunofluorescence microscopy detection (Merifluor) recognized epitopes on the oocyst wall of *C. canis*; therefore, morphometric features were not helpful in differentiating oocysts of *C. canis* from oocysts of other *Cryptosporidium* spp.

The difference in susceptibility to infection observed in neonatal BALB/c test mice that received oocysts from the dog versus control mice that received oocysts from the bovine source was clearly evident in ileum examined microscopically 4 days after inoculation. None of 4 mice (group 1) that received oocysts from the dog had detectable cryptosporidial parasites, whereas all 4 control mice were heavily infected (Table II). When group 2 of BALB/c mice were similarly tested and tissues were examined 6 days after inoculation, a few developmental stages were observed in mice that had received oocysts from the dog, suggesting that either oocysts of *C. canis* took longer to develop or that a small number of *C. parvum* bovine genotype were present in the inoculum. Mature C57 mice did not excrete oocysts after receiving oocysts from dog or bovine sources (group 4) unless they were immunosuppressed (group 3, Table II). Oocysts from all immunosuppressed mice were *C. parvum* bovine genotype, suggesting that oocysts from the dog contained both *C. canis* and *C. parvum* bovine genotype and that the latter was infectious for the mice, but *C. canis* was not. These findings, as well as finding a trace amount of DNA corresponding with *C. parvum* bovine genotype in oocysts from the dog, strongly suggested that the dog oocysts consisted of a mixture of predominantly *C. canis* oocysts with a trace of *C. parvum* bovine genotype oocysts. It is possible that the im-

munosuppressed dog also acquired infection with the *C. parvum*, bovine genotype in addition to the *C. canis*, in the research facility. This is not unlike eimerian infections in poultry and cattle, where several species can produce concurrent infections in a single host. Mixed infections both in the dog and in calves 1 and 2 with *C. parvum* bovine genotype and *C. canis* indicates that within each of these hosts the isolates remained genetically distinct. The great reduction in the percentage of *C. canis* oocysts excreted by calf 2 versus calf 1 is not entirely clear but may reflect the variable volume of feces collected each day from each calf, with more oocysts of one species excreted on a day when few feces were collected; interspecies competition within the intestinal tract, resulting in greater fecundity of 1 species; or other factors. The excretion of *C. canis* oocysts by calf 3 that received oocysts from a human source confirmed the infectivity of *C. canis* in 3 bovine hosts.

Results of genetic characterization support the classification of oocysts from the dog as a separate species, *C. canis*. The genetic difference in the SSU rRNA and HSP 70 genes between *C. canis* and the *C. parvum* bovine, mouse, and human genotypes is greater than or comparable to the differences between established species, such as *C. parvum* and *C. meleagridis*, *C. parvum*, and *C. wrairi*, or *C. andersoni* and *C. muris*, *C. muris*, and *C. serpentis* (Figs. 6A, B). For example, the genetic distances between *C. canis* and *C. parvum* (bovine genotype) was 3.29% for SSU rRNA and 15.54% for HSP 70 (Table IV). These are greater than the distances between *C. parvum* (bovine genotype) and *C. wrairi* (0.60% for SSU rRNA and 1.82% for HSP 70), *C. parvum* and *C. meleagridis* (1.32% for SSU rRNA and 4.23% for HSP 70), or *C. muris* and *C. serpentis* (2.32% for SSU rRNA and 4.35% for HSP 70). This is also reflected

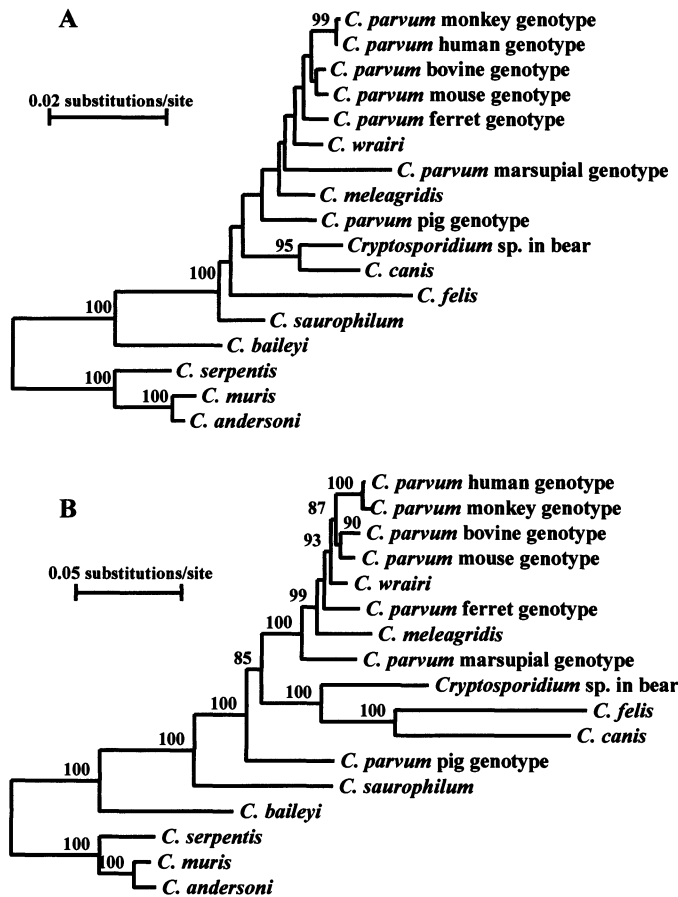


FIGURE 6. Phylogenetic relationship (neighbor-joining tree based on Kimura 2-parameter analysis) between *Cryptosporidium* species and *C. parvum* genotypes inferred from nucleotide sequences of the SSU rRNA (Fig. 5A) and from sequences of the 70-kDa heat shock protein (Fig. 5B). Modified from Xiao et al. (1996); Xiao, Limor et al. (2000); and Sulaiman et al. (2000).

in the phylogenetic analysis of the sequence data. Neighbor-joining trees constructed based on nucleotide sequences of SSU rRNA and HSP 70 (Figs. 6A, B) placed *C. canis* outside most parasites currently classified as *C. parvum* (Xiao, Escalante, Yang et al., 1999; Morgan et al., 2000; Sulaiman et al., 2000). Another indication of the genetic uniqueness of *C. canis* is the GC content of the HSP 70 gene (Sulaiman et al., 2000). Although most *Cryptosporidium* characterized so far are AT-rich in the HSP 70 gene (58–66% of A or T), *C. canis* and *C. felis* are the only species of *Cryptosporidium* with balanced GC content (48.2 and 51.0% of A or T for *C. canis* and *C. felis*, respectively), providing strong support for the genetic uniqueness of *C. canis* as a valid species.

Approximately 152 species of mammals are reported to have been infected with *C. parvum*-like parasites (Fayer et al., 2000). As more and more isolates of what appear to be *C. parvum* are identified and determined by molecular analysis to differ from one another genetically, it appears increasingly less accurate, or even impossible in some cases, to characterize any isolate by morphometric characteristics and host specificity alone. There have been 8 genotypes of *C. parvum* identified as human, monkey, bovine, pig, marsupial, mouse, ferret, and bear (Morgan et

al., 1999; Xiao, Escalante, Yang et al., 1999; Xiao, Morgan et al., 2000). Without genetic analysis, it is impossible to accurately predict host specificity or infectious potential of a specific isolate of *Cryptosporidium*. In a study in which 6-wk-old Beagle dogs were fed oocysts of bovine origin, all dogs became infected and shed oocysts in feces (Lloyd and Smith, 1997). In other studies, healthy pups experimentally inoculated with *C. parvum* (genotype unknown) developed transient diarrhea and shed oocysts (Wilson and Holscher, 1983; Augustin-Bichl et al., 1984; Sisk et al., 1984). In yet another study, pups inoculated with oocysts from an infected human (genotype unknown) became infected and shed oocysts (Current et al., 1983). Humans, with compromised immunity in some cases, and HIV-negative children, have served as hosts for 5 genetically different types (Xiao et al., 2001). These include *C. parvum* human and bovine genotypes and *C. canis*, as well as *C. meleagridis* and *C. felis* (Morgan et al., 1999; Pieniazek et al., 1999; Morgan, Weber et al., 2000; Xiao, Limor et al., 2000). The foregoing observations lead us to conclude that our ability to identify and subsequently understand the epidemiology of organisms within the genus *Cryptosporidium* has been severely limited. It is apparent that the number of species, subspecies, genotypes, or other designations of organisms with indistinguishable oocysts but unique genetic and biological features are hidden under the umbrella of *C. parvum*. In an effort to bring clarity to an increasingly complex subject, it is prudent to identify as clearly as possible each genetically and biologically unique member of this genus. Therefore, the name *Cryptosporidium canis* is designated for isolates from dogs that share the same genetic and biologic characteristics as the organisms described in the present study.

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