

## *Cryptosporidium* spp. in Domestic Dogs: the “Dog” Genotype

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Received 13 September 1999/Accepted 1 February 2000

**Genetic and phylogenetic characterization of *Cryptosporidium* isolates at two loci (18S rRNA gene and heat shock gene) from both Australian and United States dogs demonstrated that dog-derived *Cryptosporidium* isolates had a distinct genotype which is conserved across geographic areas. Phylogenetic analysis provided support for the idea that the “dog” genotype is, in fact, a valid species.**

Protozoan parasites of the genus *Cryptosporidium* are found worldwide in over 170 different host species (19). *Cryptosporidium parvum* causes the majority of mammalian infections. In the immunocompetent host, infection is self-limiting, lasting from a few days to 3 weeks, with possible morbidity in young animals (6). In the immunocompromised host, infection may result in chronic debilitating diarrhea with dehydration, malabsorption, wasting, and death (6).

There have been few reports of *Cryptosporidium* infection in dogs, with the majority of cases involving pups less than 6 months of age. The first evidence of cryptosporidiosis in dogs was reported in 1981 by Tzipori and Campbell, who detected *Cryptosporidium* antibodies in 16 of 20 canine serum samples (27). Wilson et al. reported the first clinical case of canine cryptosporidiosis 2 years later, when life cycle stages characteristic of *Cryptosporidium* were identified in a 1-week-old pup suffering from acute diarrhea (28).

Simpson et al. examined 101 fecal samples collected from dogs at boarding kennels in Scotland and found that all were negative for *Cryptosporidium* (22). Similarly, Bugg et al. screened 421 fecal samples from dogs in Western Australia, using both microscopy and PCR, none of which were positive for *Cryptosporidium* oocysts (1). In contrast, 2% of stray dogs sampled in California (4), 1% of specimens from seven public parks in Scotland (9), and 1.8% of samples from urban dogs and 9.2% of samples collected from public parks in Hobart, Tasmania, were positive for *Cryptosporidium* oocysts (13). Johnston and Gasser screened fecal samples from dogs in the Geelong and Melbourne in Australia and reported *Cryptosporidium* prevalences ranging from 0.7 to 19.6% (10).

There are up to 10 valid named *Cryptosporidium* species. These include *C. parvum* from humans and many mammals, *C. muris* from rodents, *C. andersoni* from ruminants, *C. felis* from cats, *C. wrairi* from guinea pigs, *C. meleagridis* and *C. baileyi* from birds, *C. serpentis* and *C. saurophilum* from reptiles, and *C. nasonum* from fish (6, 11, 29). There is strong evidence that the most widely studied species, *C. parvum*, is not a uniform spe-

cies but consists of numerous distinct genotypes (14, 15, 16, 17, 29).

*Cryptosporidium* oocysts excreted by dogs are morphologically similar to those of *C. parvum* and have therefore been assumed to be zoonotic. However, recent genetic evidence suggests that dogs harbor a *Cryptosporidium* type genetically distinct from that detected in immunologically competent humans (29). The aim of this study was to compare dog-derived *Cryptosporidium* isolates from different geographic locations at two genetic loci (18S rRNA gene [rDNA] and heat shock gene *Hsp-70*) and to conduct a full phylogenetic analysis of dog-

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KSU1      ATTTAGGTACCACATACTCATGTGTGGTGTATGGAGAAACGATACTGTGA
C1         ATTTAGGTACCACATACTCATGTGTGGTGTATGGAGAAACGATACTGTGA
H1        ATTTAGGTACCACATACTCATGTGTGGTGTATGGAGAAACGATACTGTGA
K1        ATTTAGGTACCACATACTCATGTGTGGTGTATGGAGAAACGATACTGTGA
AusDog1   ATTTAGGTACCACATACTCATGTGTGGTGTATGGAGAAACGATACTGTGA
USADog2   ATTTAGGTACCACATACTCATGTGTGGTGTATGGAGAAACGATACTGTGA
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KSU1      GATATGTTCCAAACGATCAAGGTAACCGTACAACCCATCATATGTAGC
C1         GATATGTTCCAAACGATCAAGGTAACCGTACAACCCATCATATGTAGC
H1        GATATGTTCCAAACGATCAAGGTAACCGTACAACCCATCATATGTAGC
K1        GATATGTTCCAAACGATCAAGGTAACCGTACAACCCATCATATGTAGC
AusDog1   GATATGTTCCAAACGATCAAGGTAACCGTACAACCCATCATATGTAGC
USADog2   GATATGTTCCAAACGATCAAGGTAACCGTACAACCCATCATATGTAGC
*****

KSU1      ATTTACAGAACTGAGCGTCTTATTTGGTGTATGCGCAAAGAACAAGTTG
C1         ATTTACAGAACTGAGCGTCTTATTTGGTGTATGCGCAAAGAACAAGTTG
H1        ATTTACTGAAACTGAGCGTCTTATTTGGTGTATGCGCAAAGAACAAGTTG
K1        ATTTACAGAACTGAGCGTCTTATTTGGTGTATGCGCAAAGAACAAGTTG
AusDog1   TTTCACAGAGACAGAGCGTCTTATTTGGTGTATGCGCAAAGAACAAGTTG
USADog2   TTTCACAGAGACAGAGCGTCTTATTTGGTGTATGCGCAAAGAACAAGTTG
*****

KSU1      CTAGAAACCCAGAAAATACAGTTTTTGGTGTATGCTAAACGCTTATTGGTAGA
C1         CTAGAAACCCAGAAAATACAGTTTTTGGTGTATGCTAAACGCTTATTGGTAGA
H1        CTAGAAATCCAGAAAACAGTTTTTGGTGTATGCTAAACGCTTATTGGTAGA
K1        CTAGAAATCCAGAAAATACAGTTTTTGGTGTATGCTAAACGCTTATTGGTAGA
AusDog1   CTAGAAACCCAGAAAATACAGTTTTTGGTGTATGCTAAACGCTTATTGGTAGA
USADog2   CTAGAAACCCAGAAAATACAGTTTTTGGTGTATGCTAAACGCTTATTGGTAGA
*****

KSU1      AAGTTTGATGATCAGGCAGT
C1         AAGTTTGATGATCAGGCAGT
H1        AAGTTTGATGATCAGGCAGT
K1        AAGTTTGATGATCAGGCAGT
AusDog1   AAGTTTGATGATCAGGCAGT
USADog2   AAGTTTGATGATCAGGCAGT
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FIG. 1. Alignments of *Hsp-70* sequence data from various *Cryptosporidium* isolates. Genotypes: KSU1 and C1, cattle; H1, human; K1, marsupial; AusDog1 and USADog2, dog. Asterisks indicate identical bases.

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TABLE 1. Dog-derived *Cryptosporidium* isolates used in this study

Isolate code	Host age	Host sex, breed	Geographic origin	Source <sup>a</sup>
AusDog1	9 wk	Male, bullmastiff	Queensland, Australia	MU
AusDog2	11 yr	Female, Akita	Western Australia	MU
AusDog3	22 wk	Male, schnauzer	Western Australia	MU
USADog1	4 mo	Female, ND <sup>b</sup>	Georgia	CDC
USADog2	4 mo	Female, ND	Georgia	CDC
USADog3	4 mo	Female, ND	Georgia	CDC
USADog4	2 yr	Female, ND	Georgia	CDC
USADog5	>1 yr	Female, mixed	United States	USDA

<sup>a</sup> MU, Murdoch University, Western Australia; CDC, Centers for Disease Control and Prevention, Atlanta, Ga.; USDA, U.S. Department of Agriculture, Beltsville, Md.

<sup>b</sup> ND, not determined.

derived isolates at the 18S rDNA locus, as well as a wide range of other *Cryptosporidium* isolates or species at the same locus, in order to more clearly understand the species status of the “dog” genotype.

The sources of the dog-derived *Cryptosporidium* isolates studied are listed in Table 1. *Cryptosporidium* oocysts were initially identified using a malachite green negative strain (5) and then concentrated from intestinal scrapings and purified using phosphate-buffered saline-ether sedimentation and Ficoll gradient centrifugation (12). Dog-derived oocysts from the United States were identified by screening of fecal specimens with an immunofluorescence kit (Merifluor, Cincinnati, Ohio). Oocysts were purified by sucrose-percoll centrifugation.

DNA was extracted from purified oocysts as previously described (14), and a 713-bp region of the 18S rDNA was amplified and sequenced using the primers 18S-SF (forward primer; 5' AGTCATAGTCTTGTCTCAAAGATT 3') and 18SiR (reverse primer; 5' CTGCTTTAAGCACTCTAATTTTC 3') as previously described (18). This region encompasses the 5' end of the 18S rDNA and includes a variable region which has previously been shown to be capable of discriminating among all *Cryptosporidium* species, as well as multiple genotypes within *C. parvum* (14, 15, 17, 29). A 311-bp region of the heat shock gene *Hsp-70* was also amplified using the forward primer HSPF1 (5'-ATG TCT GAA GGT CCA GCT ATT GGT ATT

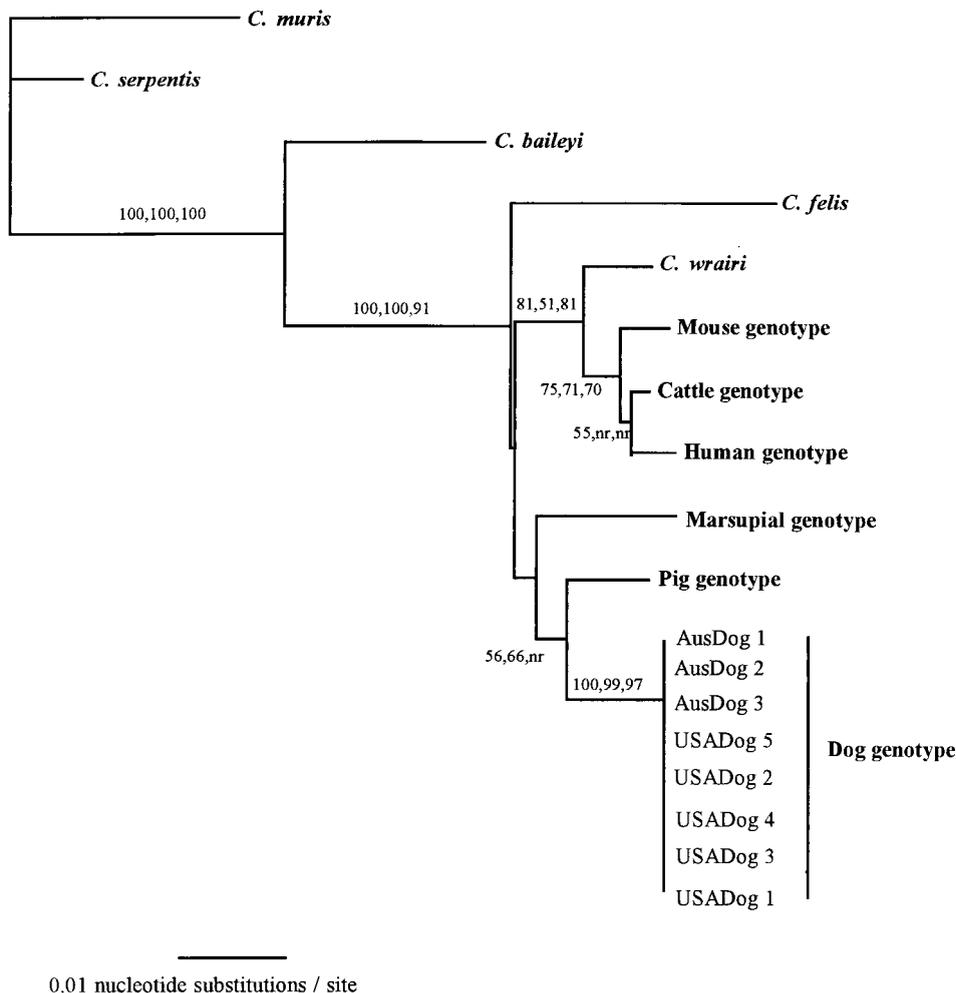


FIG. 2. Evolutionary relationships of *Cryptosporidium* isolates inferred by NJ analysis of Tamura-Nei distances calculated from pairwise comparisons of 18S rDNA sequences. Percentages of bootstrap support (>50%) from 1,000 replicate samples (analyzed by the NJ, parsimony, and maximum-likelihood methods, respectively) are indicated at the left of the supported node. nr, node not recovered by method. Additional *Cryptosporidium* 18S rDNA sequences obtained from GenBank were bovine *C. parvum* isolate C1 (AF108864), human *C. parvum* isolate H7 (AF108865), mouse genotype *C. parvum* isolate M24 (AF108863), a pig genotype isolate (AF108861), a marsupial genotype isolate (AF108860), *C. wrairi* (AF115378), *C. felis* (AF108862), *C. baileyi* (AF093495), and *C. serpentis* (AF108866).

GA-3') and the reverse primer HSPR3 (5' GAT TGG CTT RTC CTT YGG RCC 3'). DNA was amplified using 2 mM MgCl<sub>2</sub> and 45 rounds of amplification at an annealing temperature of 52°C.

Nucleotide sequences were aligned using Clustal X (25). Phylogenetic trees were constructed by three methods. Distance-based neighbor-joining (NJ) analysis and parsimony analysis were performed using PAUP, version 4.0b2 (Sinauer Associates, Sunderland, Mass.), and maximum-likelihood analyses were performed using PUZZLE, version 4.1 (24). NJ analyses were conducted using Tamura-Nei distance estimates. Parsimony analyses were conducted using either the branch and bound or heuristic search option of PAUP\*. Bootstrap analyses were conducted using 1,000 replicates. Phylograms were drawn using the TreeView program (20).

Sequence analysis of a short region of the *Hsp-70* gene revealed that dog-derived *Cryptosporidium* isolates were identical to each other but genetically distinct from other genotypes of *C. parvum*. Representative sequences are listed in Fig. 1.

Similarly, all dog-derived *Cryptosporidium* isolates from the United States and Australia also possessed identical 18S rDNA sequences. The DNA sequences from these isolates were 98.3 to 97% similar to the other *C. parvum* sequences and 95.9 to 91.9% similar to those of other *Cryptosporidium* species. Phylogenetic analysis of the sequence data (Fig. 2, NJ tree illustrated) placed all of the *C. parvum* isolates, as well as *C. felis* and *C. wrairi*, into the same cluster. This grouping was strongly supported by distance-based NJ, parsimony, and maximum-likelihood analyses. As previously reported (17, 29), *C. wrairi* grouped with the mouse, human, and cattle genotypes. The isolates of *C. parvum* from dogs formed a broad cluster with the pig and marsupial genotypes. However, this grouping was weakly supported by the distance-based NJ and parsimony methods and not recovered by maximum-likelihood analysis. A consensus tree (Fig. 3) is presented to illustrate the groupings that are well supported by the analyses.

In this study, *Cryptosporidium* was detected in two immunologically stressed Australian domestic dogs. No clinical data were available for the third Australian dog. The first Australian dog-derived isolate (AusDog1), was from a 9-week-old male bullmastiff admitted to the Murdoch University Veterinary Hospital with vomiting, lethargy, and diarrhea. Serum analysis indicated a recent canine parvovirus infection (3). The dog had recently arrived in Western Australia from Queensland. The second Australian dog (AusDog2) was an 11-year-old female spayed Akita. This dog was receiving treatment for immune-mediated thrombocytopenia. The dog developed hemorrhagic diarrhea, anemia, and severe hydronephrosis with acute pyonephrosis and was euthanized. With both dogs, low numbers of *Cryptosporidium* were detected in the feces. The U.S. dogs were asymptomatic.

Previous documented cases of canine cryptosporidiosis have involved symptomatic and asymptomatic animals, often with other concurrent infections. Sisk et al. (23) reported histological evidence of cryptosporidiosis in two 6-week-old pups, with one pup's death attributed to the pesticide toxophene and that of the other attributed to unspecified causes. Fukushima and Helman (7) reported an incidental finding of *Cryptosporidium* in a 3-month-old pup with canine distemper virus. Similarly, Turnwald et al. (26) detected *Cryptosporidium* oocysts in the feces of a 6-month-old female pup with canine distemper virus. In both cases, it was speculated that canine distemper caused immunosuppression, leading to increased susceptibility to cryptosporidial infection. The first case of chronic cryptosporidiosis was reported in an adult dog which had a 2-year history of intermittent diarrhea and weight loss (8). Immunosuppres-

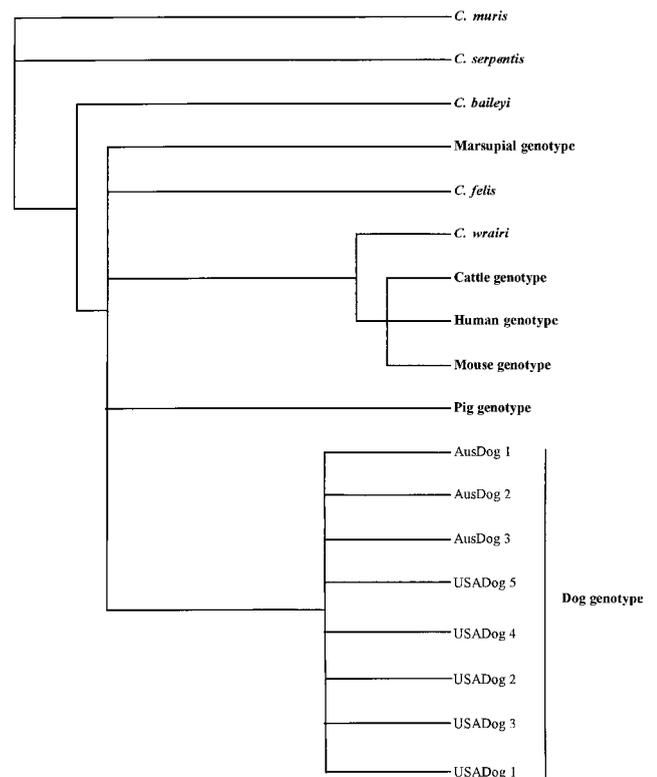


FIG. 3. Parsimony consensus tree.

sion, the cause of which could not be determined, was implicated as a factor increasing the susceptibility of the animal to infection. In the present study, the fact that AusDog1 and -2 were immunosuppressed appeared to predispose them to the occurrence of cryptosporidiosis. The immune status of the remaining dogs is unknown.

To date, seven distinct genotypes have been identified within what is currently recognized as *C. parvum*: a human genotype found only in humans; a cattle genotype found in livestock (cattle, sheep, and goats), which appears to be zoonotic, as it has been found in humans; as well as pig, marsupial, mouse, ferret, and, more recently, dog genotypes (14-17, 29).

Genetic analysis of both the 18S rDNA and *Hsp-70* loci indicated that both Australian dogs were infected with a distinct *Cryptosporidium* genotype that appears to be conserved across geographic areas, as dog-derived *Cryptosporidium* isolates from the United States were identical to the Australian dog isolates at both loci.

Phylogenetic analysis of 18S rDNA sequence data obtained from dog-derived *Cryptosporidium* isolates from Australia and the United States, as well as other *Cryptosporidium* genotypes and species, revealed the dog genotype to be very distinct. The genetic similarity between the dog and the human and cattle genotypes was 97%. This is on a par with the genetic similarity between two accepted *Cryptosporidium* species, namely *C. muris* and *C. serpentis* (97%), and less than the similarity among *C. parvum* (cattle and human), *C. wrairi*, and *C. meleagridis* (99 to 98.4%). The level of genetic divergence between the dog genotype and other *Cryptosporidium* genotypes, combined with its apparently host-adapted nature, provides support for the idea that the dog genotype is, in fact, a separate species.

The infectivity of canine isolates for immunocompetent hu-

mans has not been demonstrated, although it has been shown that dogs can be experimentally infected with oocysts of human origin (2). It is speculated that humans may acquire infection from naturally infected dogs. Zoonotic transmission from a dog was suspected in one case when a veterinary student working in a ward where an infected dog was being cared for developed acute self-limiting diarrhea and *Cryptosporidium* oocysts were identified in her feces (8). In the present study, in the case of AusDog1, neither the dog's littermate nor the owners or veterinary personnel were *Cryptosporidium* positive, although opportunity for cross-infection existed. The dog genotype has, however, recently been identified in human immunodeficiency virus-infected human patient in the United States (21), and clearly, further studies are required in order to understand the full public health significance of the *Cryptosporidium* dog genotype, as well as other genotypes and species, for immunocompromised hosts.

In conclusion, the results of this study indicate that dogs harbor a genetically distinct dog-adapted *Cryptosporidium* strain which is conserved across geographic areas and which may well be a valid species. Further genetic and biological studies are required to fully determine the prevalence, transmission dynamics, and species status of the *Cryptosporidium* dog genotype.

We thank Aileen Elliot for microscopy assistance.

This study was supported by the Public Health Research and Development Committee (PHRDC) of the National Health and Medical Research Council of Australia. U. Morgan is a PHRDC Research Fellow.

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