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CRYPTOSPORIDIUM SUIS N. SP. (APICOMPLEXA: CRYPTOSPORIDIIDAE) IN PIGS (*SUS SCROFA*)

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ABSTRACT: Molecular and biological characteristics of a new species of *Cryptosporidium* from the feces of pigs (*Sus scrofa*) is described. Oocysts are structurally indistinguishable from those of *Cryptosporidium parvum*; they are passed fully sporulated, lack sporocysts, and measure 4.9–4.4 μm (mean = 4.6 μm) × 4.0–4.3 μm (mean = 4.2 μm); length to width ratio 1.1 (n = 50). *Cryptosporidium suis* is not transmissible to nude mice and is poorly infectious for cattle. Molecular and phylogenetic analyses at the 18S ribosomal RNA, heat shock protein 70, and actin gene loci demonstrate *C. suis* to be genetically distinct from all known species and genotypes of *Cryptosporidium*, and thus is named as *Cryptosporidium suis*.

At present, 13 species of *Cryptosporidium* are regarded as valid on the basis of differences in genetics, oocyst morphology, and site of infection: *C. muris* in rodents; *C. andersoni* in cattle; *C. parvum* in ruminants and humans; *C. wrairi* in guinea pigs; *C. hominis* in humans; *C. meleagridis*, *C. baileyi*, and *C. galli* in birds; *C. serpentis* and *C. saurophilum* in snakes and lizards; *C. molnari* in fish; *C. felis* in cats; and *C. canis* in dogs (Fayer et al., 2000, 2001; Alvarez-Pellitero and Sitja-Bobadilla, 2002; Morgan-Ryan et al., 2002; Ryan, Xiao et al., 2003).

Traditionally, taxonomic classification for coccidia and other protozoa has been based on phenotypic characters such as morphological features and host specificity (Fayer et al., 2000). However, morphology has been shown to be an unreliable means of delineating species within *Cryptosporidium* (Fall et al., 2003). This is in part because of the fact that oocysts of *Cryptosporidium* spp. are among the smallest exogenous stages of the apicomplexans and also because of the lack of distinguishing morphological characters, as there are only 2 characters that can be analyzed (length by width and shape index). *Cryptosporidium parvum* is the most widely studied species, and whereas no morphological difference has been identified, there is now strong evidence that there are numerous genetically distinct genotypes within the *C. parvum* group, which are likely to be cryptic species (Xiao et al., 1999; Morgan-Ryan et al., 2002; Xiao, Sulaiman et al., 2002; Xiao et al., 2003).

Recent genetic and biological characterization studies have identified 2 distinct apparently host-adapted genotypes of *Cryptosporidium* in pigs, i.e., the *Cryptosporidium* pig genotype I and pig genotype II (Morgan et al., 1998; Pereira et al., 1998; Morgan et al., 1999; Sulaiman et al., 2000, 2002; Enemark et al., 2003; Ryan, Samarasinghe et al., 2003). In the present study, we present evidence that pig genotype I is in fact a valid species and propose the name *Cryptosporidium suis* (pig genotype I).

MATERIALS AND METHODS

Sources of parasite isolates

Isolates were obtained from *Cryptosporidium*-positive fecal samples from 3- to 5-wk-old pigs (see Table I).

Deoxyribonucleic acid extraction, polymerase chain reaction, and sequence analyses

Oocysts were purified, and deoxyribonucleic acid (DNA) was extracted as described previously (Xiao et al., 1999). Fragments of the 18S ribosomal RNA (rRNA) (~830 bp), heat shock protein (HSP) 70 (~325 bp), and actin (~1,095 bp) genes were amplified by polymerase chain reaction (PCR) as described previously (Xiao et al., 1999; Morgan et al., 2001; Sulaiman et al., 2002). PCR products were purified using Qiagen spin columns (Qiagen, Valencia, California) or Wizard PCR Prep Kit (Promega, Madison, Wisconsin) and sequenced in both directions on an ABI377 or ABI3100 Autosequencer using an ABI BigDye™ Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions. Each isolate was sequenced at least twice with independent PCR products.

Phylogenetic analyses

Nucleotide sequences obtained from this study were aligned against each other and against those obtained previously using Clustal X (Thompson et al., 1997); (sequence alignments can be obtained from the authors upon request). *Plasmodium falciparum* was used as an outgroup for HSP 70 (GenBank M19753), 18S rRNA (Genbank M19172), and actin (Genbank M19146) analyses. Distance-based analyses of 18S rRNA, HSP 70, and actin sequences were performed using MEGA version 2.1 (Kumar et al., 2001). Neighbor-joining (NJ) trees were constructed on the basis of genetic distances calculated with the Tamura–Nei model.

Parsimony and maximum-likelihood (ML) analyses were used to validate the phylogenetic relationship inferred by the NJ analyses. Parsimony analyses were performed by PAUP* (version 4.0b2), using the heuristic search option. The following settings were used for heuristic parsimony analysis: all characters were treated as unordered with equal weight, and gaps were treated as missing; starting trees obtained by stepwise addition; addition sequence = simple; branch swapping algorithm = TBR. For HSP 70 sequences, ML analysis (performed by PAUP* [version 4.0b2]) using an heuristic search was conducted using the following settings: HKY85 model settings with 2 substitution types;

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TABLE I. Isolates of *Cryptosporidium* used in this study.

Isolate code	Age of host (wk)	Geographic origin
SP1	3–4	Switzerland
WAP1	3–4	Western Australia
WAP5	3–4	Western Australia
WAP8	3–4	Western Australia
WAP12	5	Western Australia

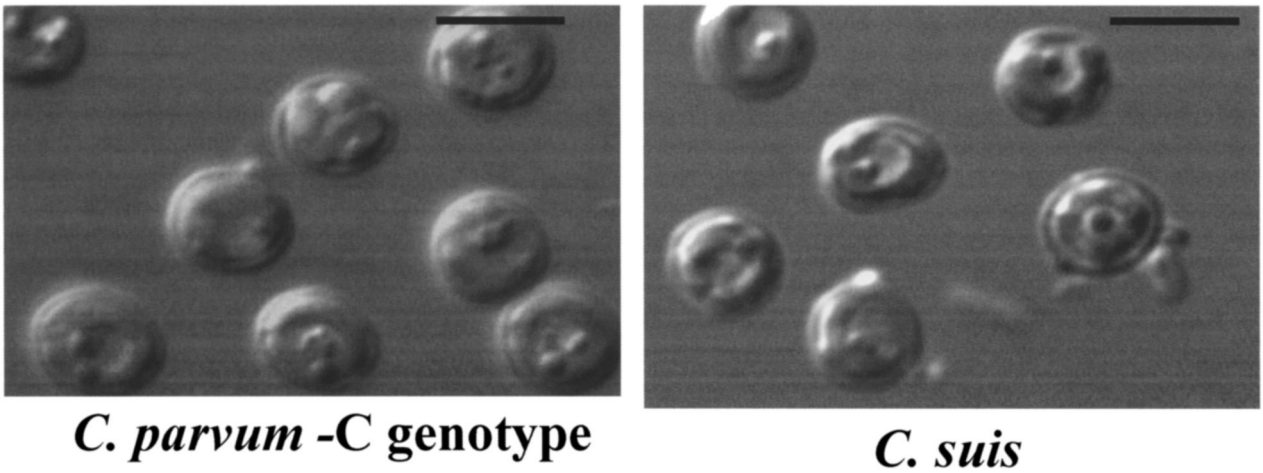

FIGURE 1. Nomarski interference microscopy of *Cryptosporidium* oocysts of *C. parvum* and *C. suis* n. sp. Bar = 5 μm.

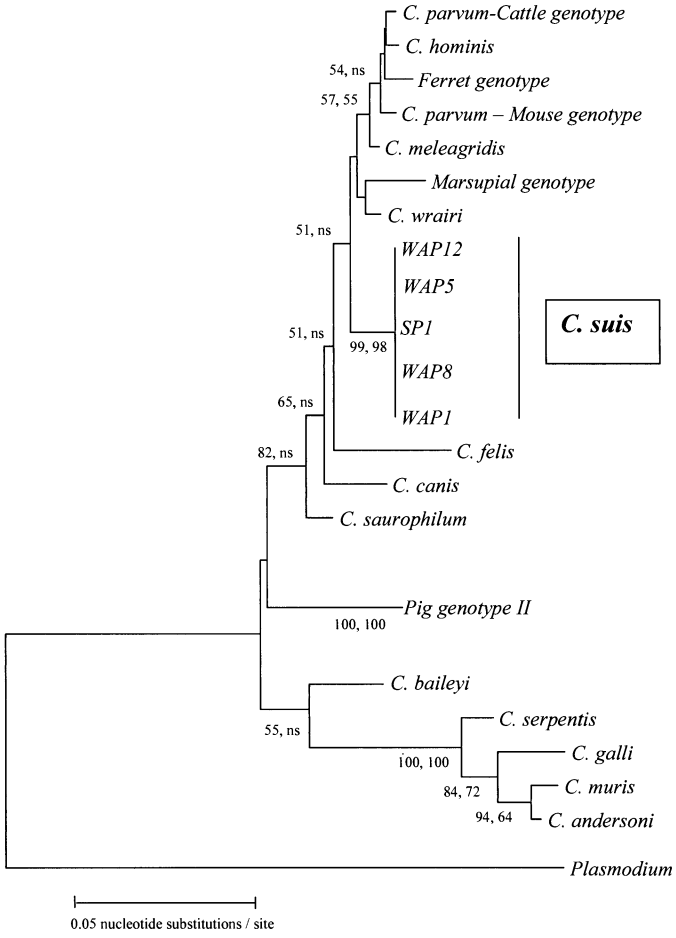

FIGURE 2. Evolutionary relationships of *Cryptosporidium* isolates inferred by NJ analysis of Tamura–Nei distances calculated from pairwise comparisons of the 18S rDNA sequences. Percentage bootstrap support (>50%) from 1,000 replicate samples (analyzed by NJ and parsimony methods, respectively) is indicated at the left of the supported node. ns = node not supported by method.

transition–transversion ratio estimated by ML; empirical base frequencies used; starting branch lengths obtained using Rogers–Swofford method; branch-length optimization by 1-dimensional Newton–Raphson with pass limit = 20; starting trees obtained by stepwise addition; addition sequence = as-is; branch swapping algorithm = TBR. Bootstrap analyses for distance-based and parsimony methods were conducted using 1,000 replicates to assess the reliability of inferred tree topologies. Bootstraps were conducted for ML analysis using 1,000 replicates for the HSP 70 analysis and 108 replicates (reduced because of computing and time constraints) for the actin analysis.

Nucleotide sequence accession numbers

The nucleotide sequences of the 18S ribosomal DNA (rDNA), HSP 70, and actin gene sequences of *Cryptosporidium* spp. isolates have been deposited in GenBank under the accession numbers AF108861, AF221533, and AF382344.

DESCRIPTION

Cryptosporidium suis (pig genotype I)

(Fig. 1)

Description: Oocysts are excreted already sporulated. They measure 4.9–4.4 μm (mean = 4.6 μm) × 4.0–4.3 μm (mean = 4.2 μm); length to width ratio 1.1 (n = 50).

Type hosts: Pigs (*Sus scrofa*).

Other hosts: There has been 1 report of this species in humans (Xiao, Bern et al., 2002).

Type locality: Perth, Western Australia.

Other localities: Cosmopolitan.

Location in host: Epithelial cells of the small and large intestine (Enemark et al., 2003).

Prepatent period: 4.8 days (range 2–9) (Enemark et al., 2003).

Patent period: 12.6 days (range 9–15) (Enemark et al., 2003).

Sporulation time: Oocysts are excreted fully sporulated.

Material deposited: A phototype of sporulated oocysts and Genbank accession numbers have been deposited in the United States National Parasite Collection (USNPC), Beltsville, Maryland. USNPC 094038.

Etymology: This species is named *Cryptosporidium suis* as it appears to be adapted to this host.

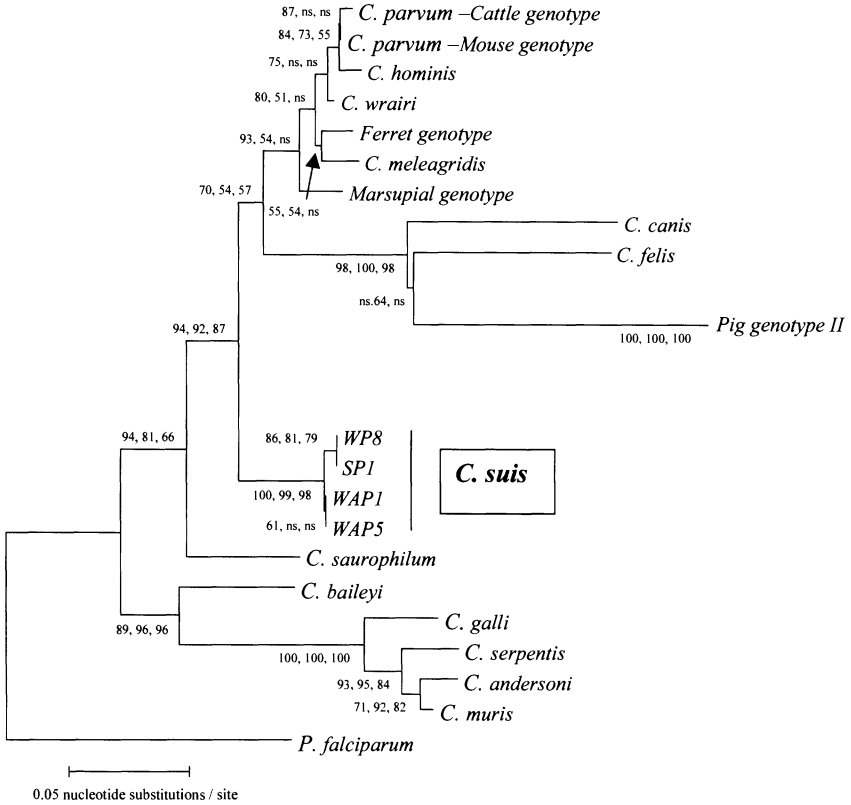

FIGURE 3. Evolutionary relationships of *Cryptosporidium* isolates inferred by NJ analysis of Tamura–Nei distances calculated from pairwise comparisons of the *hsp-70* DNA sequences. Percentage bootstrap support (>50%) from 1,000 replicate samples (analyzed by NJ, parsimony, and ML methods, respectively) is indicated at the left of the supported node. ns = node not supported by method.

Remarks

Previous studies have shown that this species is not infectious for mice (Morgan et al., 1999) and is poorly infectious for cattle (Enemark et al., 2003). Experimental infections indicated that *C. suis* is adapted to porcine hosts as demonstrated by the absence of clinical signs despite the excretion of high numbers of oocysts (Enemark et al., 2003). This is supported by another recent study of *C. suis* in Yorkshire-Landrace piglets on a pig farm northeast of Calgary, Canada. In this study, oocyst excretion was not associated with diarrhea (Guselle et al., 2003).

The 18S rDNA gene sequences were obtained from 5 *C. suis* isolates (Table I). These sequences were compared with *Cryptosporidium* sequence information obtained from GenBank. Both distance-based and parsimony analyses demonstrated that the *C. suis* isolates formed a cohesive genetic group that was distinct from all other species or genotypes of *Cryptosporidium* (Fig. 2, NJ tree illustrated). The position of *C. suis* was poorly resolved by either forms of analysis. Both methods of analysis placed *C. suis* within a cluster containing the various genotypes of *C. parvum, C. hominis, C. meleagridis, C. wrairi, C. felis, C. canis*, and *C. saurophilum*. However, the exact position of *C. suis* varied depending on the method of analysis, and there was generally poor bootstrap support for much of the tree topology, with the exception of the cluster forming *C. suis* and the cluster comprising *C. serpentis, C. galli, C. muris*, and *C. andersoni*.

Partial sequences of the *Cryptosporidium* HSP 70 gene were obtained from 4 *C. suis* isolates (Table I). These sequences were compared with *Cryptosporidium* sequence information obtained from GenBank. The phylogenetic relationships of the isolates could be better resolved compared with the results obtained using the 18S rDNA sequence data. All methods of analyses strongly supported the placement of the *C. suis* isolates into a single unique cluster, which was in agreement with the 18S rDNA analysis (Fig. 3, NJ tree illustrated). In addition, the analysis suggested some substructuring within *C. suis*, with isolates WP8 and SP1 forming a subgroup. In contrast with the 18S rDNA tree, the HSP 70 analysis placed *C. suis* external to the cluster containing the various genotypes of *C. parvum, C. hominis, C. meleagridis, C. wrairi, C. felis*, and *C. canis*. This placement was supported by all methods of analysis and received high bootstrap support (87–94%, depending on the method of analysis).

Partial actin gene sequence information was obtained from 3 *C. suis* isolates (Table I). These sequences were compared with sequences from a range of *Cryptosporidium* species and genotypes obtained from GenBank. Phylogenetic analysis was consistent with the 18S rDNA and HSP 70 sequence analyses, with the *C. suis* isolates forming a distinct cluster. NJ and ML analyses of the actin data (Fig. 4) were consistent with the 18S rDNA tree (Fig. 2), placing *C. suis* external to the cluster containing *C. hominis, C. wrairi, C. meleagridis*, and the cattle, mouse, ferret, and marsupial genotypes. Heuristic analysis identified 6 equally most parsimonious trees (not shown), 4 of which placed *C. suis* in the same position as the NJ tree.

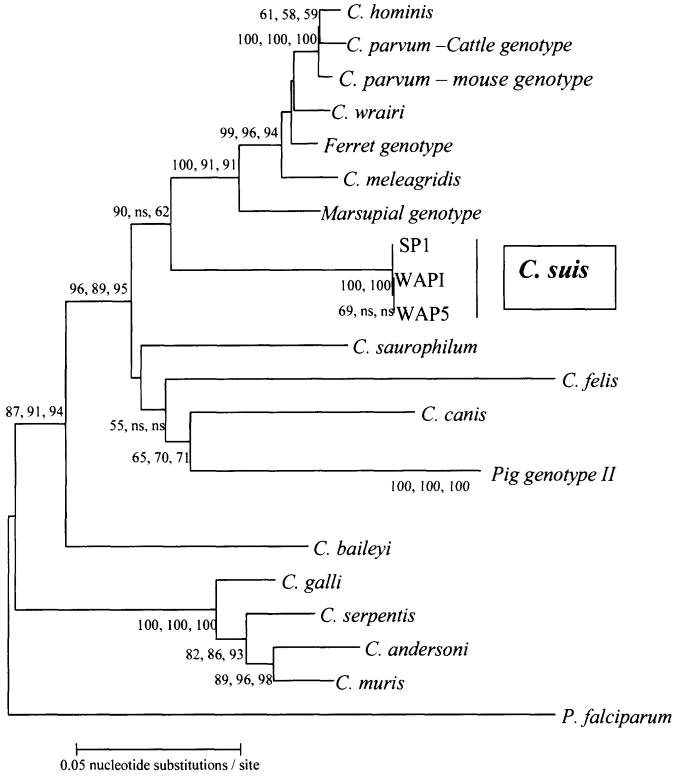

FIGURE 4. Evolutionary relationships of *Cryptosporidium* isolates inferred by NJ analysis of Tamura–Nei distances calculated from pairwise comparisons of the actin DNA sequences. Percentage bootstrap support (>50%) from 1,000 replicate samples (analyzed by NJ and parsimony methods, respectively) or 108 replicates (ML analysis) is indicated at the left of the supported node. ns = node not supported by method.

DISCUSSION

Because of the difficulties associated with the taxonomy of *Cryptosporidium* spp., several guidelines have been developed as an aid in establishing new species in this genus (Xiao et al., 2003). When naming new species of *Cryptosporidium*, 4 basic requirements should be fulfilled. First, morphometric studies of the parasite's oocysts must be undertaken. Second, the parasite must be characterized genetically. Third, there must be a demonstration of natural and, if feasible, some experimental host specificity. Finally, the study must be done in compliance with the International Code of Zoological Nomenclature. Thus, the taxonomic descriptions should constitute valid published work, and the morphological description itself and appropriate photographs should be deposited in a recognized museum, i.e., at the USNPC in Beltsville, Maryland (Xiao et al., 2003).

In the present study, *C. suis* was shown to be morphologically identical to the *C. parvum* 'cattle' genotype. However, phylogenetic analyses confirm the validity of *C. suis* at 3 independent loci. The HSP 70 data appear to give the best-resolved phylogeny for *Cryptosporidium* overall, as determined by bootstrap analysis. The topology of the HSP 70 tree is largely supported by the actin and 18S rDNA trees, with the main exception being the placement of *C. suis*. Both actin and HSP 70 datasets provide strong support for the clustering of *C. hominis, C. wrairi, C. meleagridis*, and the cattle, mouse, ferret, and marsupial genotypes.

For the 18S locus, *C. suis* shared only 97% similarity with the *C. parvum* cattle genotype and *C. hominis*. This is less than the similarity between *C. meleagridis* and the *C. parvum* cattle genotype and *C. hominis* (98.6%) and between the *C. parvum* 'cattle' genotype and *C. hominis* (99.42%). *Cryptosporidium suis* does not appear to be closely related to the novel pig genotype II because they shared only 93% similarity at the 18S locus.

There have been few studies that have examined the infectivity and pathogenicity of *C. suis* because the majority of previous studies either did not genotype the porcine isolates used or used oocysts from calves (Tzipori et al., 1981, 1982; Heine et al., 1984; Ayeni et al., 1985).

A recent study provides evidence that *C. suis* is adapted to a porcine host and causes either mild or no clinical signs in pigs (Enemark et al., 2003). The study reported the prepatent period for *C. suis* to be 4.8 days (range 2–9), compared with 3.5 days (range 2–5) for the *C. parvum* cattle genotype (Enemark et al., 2003). Pereira et al. (2002) reported the prepatent period for the cattle genotype in gnotobiotic piglets to be 5.6 days. However, it is difficult to compare results from different studies because different *C. parvum* cattle genotype isolates of *Cryptosporidium* were used in different hosts (cattle vs. pigs). It has been shown that different isolates of the same genotype can exhibit very different pathogenicity and infectivity in the same host (Okhuysen et al., 1999). In the study by Enemark et al. (2003), there were distinct differences in pathogenicity between *C. suis* and the *C. parvum* cattle genotype in pigs. For example, no mortality was seen in piglets infected with *C. suis*, whereas infection with the *C. parvum* cattle genotype was fatal in 1 of 6 piglets. The severity and duration of diarrhea was also less in pigs infected with *C. suis* (1.2 days duration vs. 3.5 days for the *C. parvum* cattle genotype). In addition, the maximum oocysts output was not detected until 12 days postinfection (dpi) in *C. suis*–infected pigs compared with 3.5 days for *C. parvum* cattle genotype–infected pigs (Enemark et al., 2003).

Guselle et al. (2003) examined oocyst shedding in pigs (n = 33) in a hog farm northeast of Calgary and reported the mean age of initial oocyst detection was 45.2 days after weaning, with the mean duration of infection being 28.7 days (Guselle et al., 2003). Genetic sequences (HSP 70) were obtained for 10 of the 33 isolates and all were *C. suis* (pig genotype I). The study reported that the mean number of oocysts shed was low and that there was no association between diarrhea and oocyst shedding (Guselle et al., 2003).

Unlike the *C. parvum* cattle genotype, *C. suis* is not infective for nude mice (Morgan et al., 1999) and is poorly infectious for cattle (Enemark et al., 2003). In the latter study, 2 attempts were made to infect calves with *C. suis*; 1 resulted in no detectable oocyst production. In the second, there were very low numbers of oocysts detected at 15 dpi but no clinical signs in the second attempt (Enemark et al., 2002). Another investigation revealed that squirrels have a separate genotype, which is very similar to *C. suis* in 18S-PCR–restriction fragment length polymorphism analysis (Atwill et al., 2001); however, sequence information is required to confirm this observation.

The genetic and biological data discussed above indicate that the differences between *C. suis* and other *Cryptosporidium* spp. are comparable with those between established species. Therefore, *C. suis* should be considered valid species.

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