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A REDESCRIPTION OF *CRYPTOSPORIDIUM GALLI* PAVLASEK, 1999 (APICOMPLEXA: CRYPTOSPORIDIIDAE) FROM BIRDS

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ABSTRACT: *Cryptosporidium galli* Pavlasek, 1999, described from the feces of birds, is redescribed with additional molecular and biological data. Oocysts are ellipsoidal, are passed fully sporulated, lack sporocysts, and measure 8.25 × 6.3 μm (range 8.0–8.5 × 6.2–6.4 μm) with a length–width ratio of 1.30 (n = 50). Oocysts are structurally similar to those of *Cryptosporidium baileyi* described from chickens, but in addition to being considerably larger than oocysts of *C. baileyi*, these oocysts infect the proventriculus in a variety of birds and not the respiratory tract. Oocysts were successfully transmitted from chickens to chickens, and morphologically similar oocysts also were observed in a variety of exotic and wild birds (Order Passeriformes, Phasianidae, Fringillidae, and Icteridae). Molecular and phylogenetic analyses at the 18S rRNA, HSP70, and actin gene loci demonstrate that this species is genetically distinct from all known species and genotypes of *Cryptosporidium* and, thus, was named *C. galli*.

Cryptosporidial infections have been reported in over 30 species of birds (O'Donoghue, 1995; Fayer et al., 1997; Sreter and Varga, 2000). However, to date only 2 avian species are accepted as valid, *Cryptosporidium baileyi* and *C. meleagridis*. *Cryptosporidium baileyi* oocysts measure 6.2 × 4.6 μm (Current et al., 1986) and have been described from broiler chickens, black-headed gulls, and quail. *Cryptosporidium meleagridis* oocysts measure 5.2 × 4.6 μm and have been described from turkeys, chickens, ducks, and, more recently, a parrot (Slavin, 1955; O'Donoghue, 1995; Morgan et al., 2000). These species have been validated on the basis of molecular differences (Xiao et al., 1999; Morgan et al., 1999, 2000, 2001; Glaberman et al., 2001) and differences in oocyst and sporozoite morphology (Lindsay et al., 1989). In addition, *C. baileyi* develops mainly in the respiratory tract (Current et al., 1986), whereas *C. meleagridis* infects the small intestine (Current et al., 1986; Pavlasek, 1994).

A recent study identified 2 new genotypes of *Cryptosporidium* in birds, i.e., a black duck genotype and a finch genotype (Morgan et al., 2001). The finch genotype was identified in a variety of finch species and was associated with clinical illness. In addition, *Cryptosporidium* sp. infection was identified only in the proventriculus, and the isolates were shown to be genetically distinct from all other species or genotypes of *Cryptosporidium* sp. at both the 18S and the HSP70 loci, indicating a potentially new avian species of *Cryptosporidium*. In that publication, the finch genotype was erroneously referred to as *Cryptosporidium blagburni* in table 1 (Morgan et al., 2001). The same parasite was previously described as a new species of *Cryptosporidium* in chickens and was named *C. galli* (Pavlasek, 1999, 2001). As in the finch genotype, this species infected only the proventriculus and was associated with clinical illness.

In the present study, morphological, biological, and molecular data are presented for *C. galli* oocysts from a variety of bird species. These oocysts are ellipsoidal, are passed fully sporulated, lack sporocysts, and measure 8.25 × 6.3 μm (range 8.0–8.5 × 6.2–6.4 μm) with a length to width ratio of 1.3 (n = 50). Although morphologically similar to *C. baileyi*, these oocysts are considerably larger, and this species infects the proventriculus and not the respiratory tract. We propose that this parasite, previously referred to as the finch genotype and named *C. blagburni* without adequate description, be identified as *C. galli* and recognized as a valid species on the basis of the characteristics proposed by Pavlasek (1999, 2001) and supported by the present data.

MATERIALS AND METHODS

Oocysts

Oocysts of *Cryptosporidium* were obtained from the feces of spontaneously infected birds (see Table I) from the Czech Republic. Fecal samples were examined and oocysts purified using routine coprological methods (Breza, 1957; Pavlasek, 1991). Oocysts were stored in 2.5% potassium dichromate at +4 C until required for molecular analysis. For each isolate, 50 oocysts were measured at ×1,000 magnification. The lengths, widths, and shape index of oocysts of *C. baileyi* (n = 50), *C. meleagridis* (n = 40), and *C. galli* (pooled values of isolate Czech-B1-25 and Czech B1-31, n = 100) were compared using an analysis of variance. Values were considered statistically different if $P < 0.05$.

Transmission studies

Oocyst inocula were prepared by washing purified oocysts with phosphate-buffered saline (pH 7.2) to remove potassium dichromate. The number of oocysts fed to birds was determined by counting on a hemocytometer. Two 9-day-old chickens and two 40-day-old chickens were each fed 3 × 10^4 oocysts. Feces were examined daily for >60 days for oocysts using routine coprological methods (Breza, 1957; Pavlasek, 1991).

Deoxyribonucleic acid extraction, polymerase chain reaction, and sequence analyses

Oocysts were purified and deoxyribonucleic acid (DNA) was extracted as previously described (Morgan et al., 1997). Fragments of the 18S rRNA (~830 bp), HSP70 (~325 bp), and actin (~1,095 bp) genes were amplified by polymerase chain reaction (PCR) as previously described (Xiao et al., 1999; Morgan et al., 2001; Sulaiman et al., 2002). PCR products were purified using Qiagen spin columns (Qiagen, Hilden, Germany) 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 in-

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

Isolate code	Host species
Czech-B1-25	Hen, *Gallus gallus*
Czech-B1-26	Hen, *Gallus gallus*
Czech-B1-27	Pine grosbeak, *Pinicola enucleator*
Czech-B1-28	Pine grosbeak, *Pinicola enucleator*
Czech-B1-31	Goldfinch, *Carduelis carduelis*
Czech-B2-3a	Capercaille, *Tetrao urogallus*
Czech-B2-3b	Capercaille, *Tetrao urogallus*
Czech-B2-3c	Capercaille, *Tetrao urogallus*

structions. 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 those obtained previously using Clustal X (Thompson et al., 1997). (Sequence alignments can be obtained from the authors on request.) *Plasmodium falciparum* was used as an out-group for HSP70 (GenBank M19753) and actin (GenBank M19146) analyses. Distance-based analyses of 18S ribosomal DNA (rDNA) and HSP70 sequences were performed using MEGA version 2.1 (Kumar et al., 2001), whereas the program TreeconW (Van de Peer and De Wachter, 1994) was used in distance-based analysis of actin sequences. Neighbor-joining trees were constructed based on genetic distances calculated with the Tamura–Nei model.

Parsimony and maximum likelihood (ML) analyses were used to validate the phylogenetic relationship inferred by the neighbor-joining analyses. For 18S rDNA and HSP70 sequences, parsimony and ML analyses were performed using PAUP* (version 4.0b2, Sinauer Associates, Sunderland, Massachusetts), using the heuristic search option of PAUP*. 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 = tree bisection–reconnection (TBR). For ML analysis, an heuristic search was conducted using the following settings: HKY85 model settings with 2 substitution types, transition or transversion ratio estimated by ML, empirical base frequencies used, starting branch lengths obtained using the 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. Only parsimony analysis was used in the supportive analysis of the actin sequences, using the program Phylip implemented in the phylogenetic package DAMBE (Xia and Xie, 2001).

Bootstrap analyses for distance-based and parsimony methods were conducted to assess the reliability of inferred tree topology, using 1,000 replicates, with the exception of the 18S rDNA parsimony analysis, which was restricted to 150 replicates because of computer memory and time constraints. It was not possible to conduct ML bootstrap analyses because of computational limitations.

Nucleotide sequence accession numbers

Nucleotide sequences of the 18S rRNA, HSP70, and actin gene sequences of *Cryptosporidium* isolates have been deposited in GenBank under the accession numbers AY168846–AY168849.

REDESCRIPTION

Cryptosporidium galli Pavlasek, 1999

(Fig. 1)

Description: Oocysts are excreted already sporulated. They measure 8.0–8.5 μm (mean = 8.25) × 6.2–6.4 μm (mean = 6.3 μm) with a length–width ratio of 1.30 (n = 50). Inside the oocyst there is a spherical residual body, 3.6–4.0 μm in size, usually containing 3 granules. Two of these granules, usually larger ones (approximately 1.6 μm in size), are positioned one against the other. The remaining granule is smaller (approximately 0.5–0.8 μm in size). The residual body is surrounded by 4 banana-shaped sporozoites (approximately 12.8–14.4 × 0.8–1.0 μm in size) (Fig. 1).

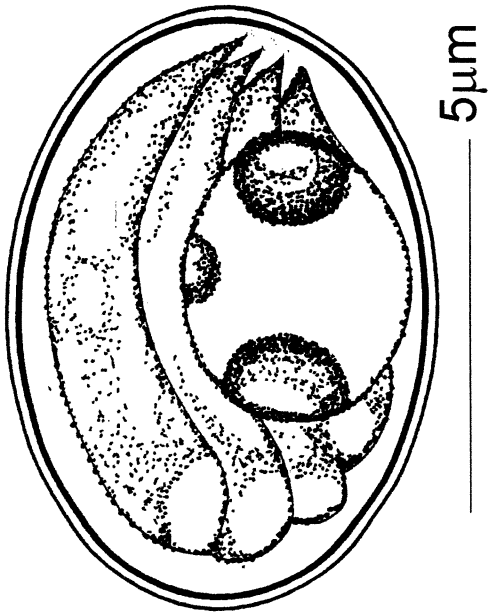

FIGURE 1. Sporulated oocyst of *Cryptosporidium galli* containing 3 granules within a spherical residual body surrounded by 4 banana-shaped sporozoites.

Type hosts: Finches (*Spermestidae* and *Fringillidae*), chickens (*Gallus gallus* f. dom.), Capercaille (*Tetrao urogallus*), Pine grosbeak (*Pinicola enucleator*).

Other hosts: Morphologically similar oocysts also were observed in a variety of exotic and wild birds including Phasianidae and Icteridae.

Type locality: Perth, Western Australia.

Other localities: Czech Republic.

Location in host: Epithelial cells of the proventriculus.

Prepatent period: Unknown.

Patent period: Unknown.

Sporulation time: Oocysts are excreted fully sporulated.

Material deposited: A line drawing of a sporulated oocyst has been deposited in the United States National Parasite Collection (USNPC), Beltsville, Maryland, and is recorded as USNPC 92689.

Etymology: This species was identified as *C. blagburni* (Morgan et al., 2001), after Dr. Byron Blagburn, who reported it in the proventriculus of a finch in 1990 (Blagburn et al., 1990). However, Pavlasek (1999, 2001) had provided a detailed description of the same parasite with the name *C. galli*. The present article redescribes *C. galli* with substantiating molecular and biological data.

Comparison of oocyst sizes

The lengths, widths, and length–width ratios of *C. baileyi, C. meleagridis*, and *C. galli* were determined to be significantly ($P < 0.05$) different from one another.

Transmission studies

Neither clinical signs nor oocysts were observed in the feces of 40-day-old chickens fed *C. galli* oocysts. For the two 9-day-old chickens, oocysts were excreted for 6 consecutive days, beginning 25 days after feeding on *C. galli* oocysts. During the next 40 days no oocyst was detected.

Sequence and phylogenetic analysis of the 18S rDNA gene

18S rDNA gene sequences were obtained from 8 *C. galli* isolates (Table I). These sequences were compared with previously obtained finch-derived *Cryptosporidium* 18S sequences (GenBank AF316623–

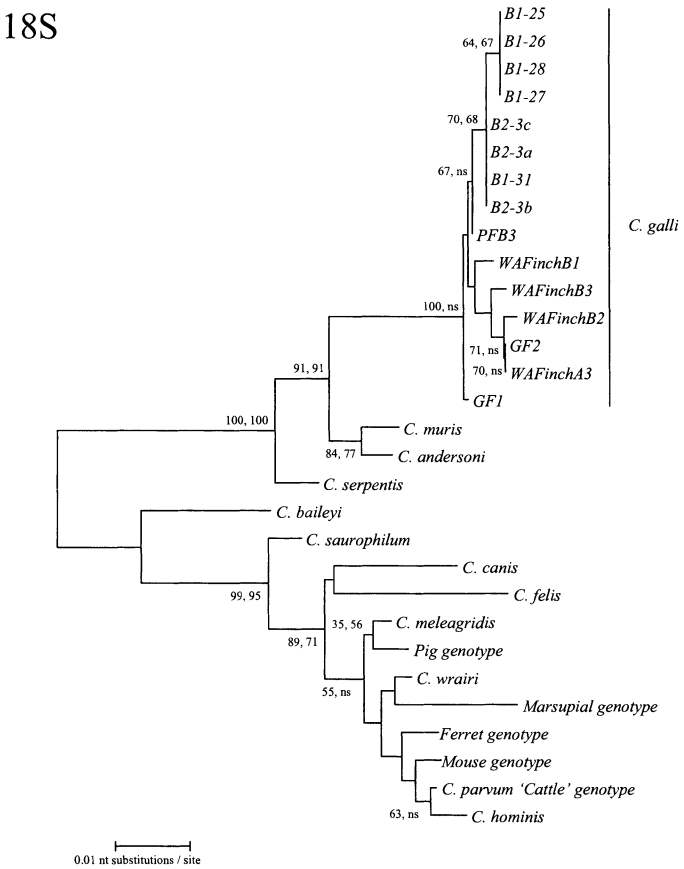

FIGURE 2. Evolutionary relationships of *Cryptosporidium* isolates inferred by neighbor-joining analysis of Tamura–Nei distances calculated from pairwise comparisons of the 18S rDNA sequences. The root for this tree was placed between *C. baileyi* and *C. saurophilum* based on the results of the HSP70 analysis. Percent bootstrap support (>50%) from 1,000 replicate samples (analyzed by neighbor-joining and parsimony methods) is indicated at the left of the supported node. ns = node not supported by method.

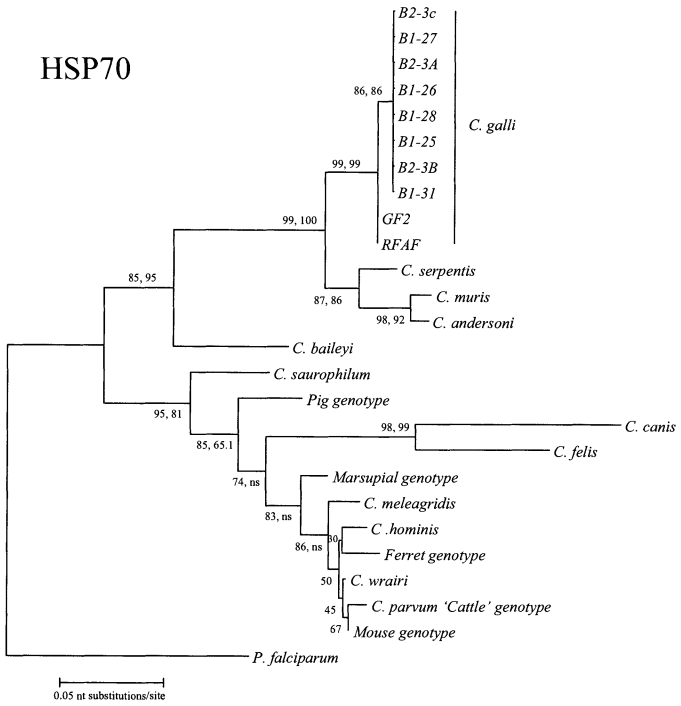

FIGURE 3. Evolutionary relationships of *Cryptosporidium* isolates inferred by neighbor-joining analysis of Tamura–Nei distances calculated from pairwise comparisons of the HSP70 DNA sequences. Percent bootstrap support (>50%) from 1,000 replicate samples (analyzed by neighbor-joining and parsimony methods) is indicated at the left of the supported node. ns = node not supported by method.

AF316629; Morgan et al., 2001) and with *Cryptosporidium* sp. sequence information obtained from GenBank as described above.

Analysis of the 18S rDNA nucleotide sequence data by distance-based (Fig. 2) ML and parsimony methods was largely consistent with the results of the HSP70 sequence.

The clustering of *C. galli* isolates with one another and the placement of this lineage relative to *C. serpentis, C. muris*, and *C. andersoni* received high bootstrap support (Fig. 2). Also, analysis identified intraspecific variation within *C. galli*. Although the bootstrap support was not high for this part of the tree, the result supported the HSP70 analysis, with the European isolates forming a separate cluster to the exclusion of the Australian isolates.

All methods identified a major cluster comprising *C. felis, C. canis, C. meleagridis, C. wrairi, C. hominis*, and the *C. parvum* marsupial, pig, cattle, mouse, and ferret genotypes. As with the *hsp* analysis, the topology for this region of the tree was not well resolved. In addition, the 18S rDNA analysis did not provide significant bootstrap support for the grouping of *C. felis* and *C. canis*. Significantly, the various genotypes of *C. parvum* were not found to be monophyletic.

Sequence and phylogenetic analysis of the HSP70 gene

Partial sequences of the *Cryptosporidium* HSP70 gene were obtained from 8 *C. galli* isolates (Table I). These sequences were compared with previously obtained, finch-derived *Cryptosporidium* HSP70 sequences (GenBank AF316632 and AF316633; Morgan et al., 2001) and with *Cryptosporidium* sequence information obtained from GenBank as described above.

Analysis of the HSP70 nucleotide sequence data by distance-based ML and parsimony methods identified *C. galli* as a lineage of *Cryptosporidium* that was separate from any described species or genotypes of *Cryptosporidium* (Fig. 3, neighbor-joining tree illustrated). Distance-based and parsimony methods identified a major cluster comprising *C. saurophilum, C. felis, C. canis, C. meleagridis, C. hominis*, and the *C. parvum* marsupial, cattle, mouse, and ferret genotypes. ML analysis (tree not shown) identified a similar division but placed the root of the tree between the nodes joining *C. saurophilum* and the pig genotype of *C. parvum*. All 3 methods clustered *C. canis* with *C. felis* and agreed on the relative positions of *C. saurophilum* and the pig genotype. *Cryptosporidium galli* formed a separate cluster that included *C. baileyi, C. serpentis, C. muris*, and *C. andersoni*. The relationships between these species were recovered by all 3 methods of analysis and strongly supported by bootstrap analysis. At this locus, there was evidence of intraspecific variation within *C. galli*, with the European isolates forming a cluster to the exclusion of the 2 Australian isolates (86% bootstrap support).

Sequence and phylogenetic analysis of the actin gene

Partial actin gene sequence information was obtained from 8 *C. galli* isolates (Table I). These sequences were compared with sequences from a range of *Cryptosporidium* species and genotypes. Sequences from the *C. galli* isolates were distinctly different from sequences from all other isolates of *Cryptosporidium*.

Phylogenetic analysis of the actin locus using distance-based and parsimony methods produced 2 major clusters, the first comprising (1) the *C. parvum* monkey, cattle, mouse, ferret, marsupial, and pig genotypes and (2) the bear genotype, *C. meleagridis, C. hominis, C. felis, C. wrairi, C. canis, C. saurophilum*, and *C. baileyi* and the second group comprising *C. galli, C. serpentis, C. muris*, and *C. andersoni* (Fig. 4, neighbor-joining tree illustrated). The grouping of *C. galli* as a separate species was strongly supported by bootstrap analysis (100%).

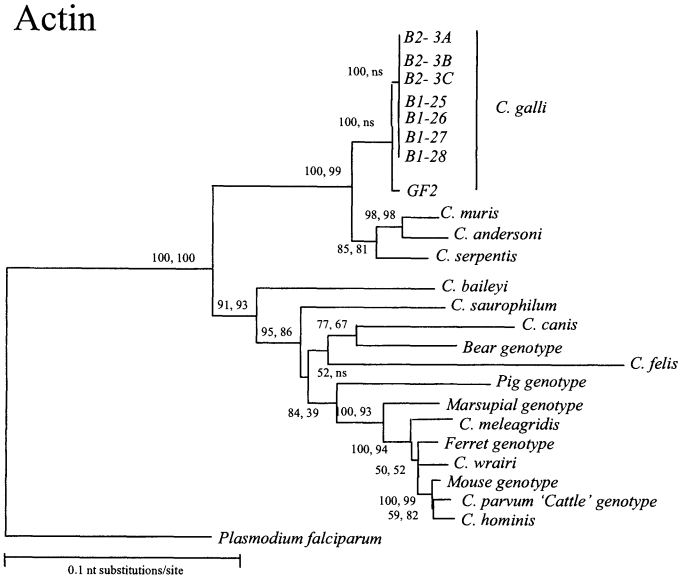

FIGURE 4. Evolutionary relationships of *Cryptosporidium* isolates inferred by neighbor-joining analysis of Tamura–Nei distances calculated from pairwise comparisons of the actin DNA sequences. Percent bootstrap support (>50%) from 1,000 replicate samples (analyzed by neighbor-joining and parsimony methods)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

DISCUSSION

Cryptosporidium galli infects the proventriculus of birds and has previously been referred to as the finch genotype and *C. blagburni* (Morgan et al., 2001). The extent of the host range and the prevalence of this species in birds have yet to be determined, but it does appear to be associated with clinical disease (Blagburn et al., 1990; Lindsay et al., 1991; Morgan et al., 2001; Pavlasek, 1999, 2001). In many cases, infected birds died after the onset of acute diarrhea (Blagburn et al., 1990; Morgan et al., 2001).

As with the *C. parvum* 'cattle' genotype that infects many species of mammals, it appears that *C. galli* can infect a wide range of birds from different orders and families. In addition, morphologically similar oocysts have been observed in a variety of exotic and wild birds, including Phasianidae, Passeriformes, and Icteridae. Further studies are required to determine the extent of the host range for *C. galli*.

Preliminary experimental transmission studies have shown that only juvenile chicks were susceptible to infection with *C. galli* and only the 9-day-old chicks and not the 40-day-old chicks became infected. Morphometrics for *C. baileyi, C. meleagridis*, and *C. galli* were statistically different ($P < 0.05$). *Cryptosporidium galli* is clearly morphologically distinct from other avian species of *Cryptosporidium* because oocysts from this species measure 8.3 × 6.3 μm, whereas *C. baileyi* oocysts measure 6.2 × 4.6 μm, and *C. meleagridis* oocysts measure 5.2 × 4.6 μm.

Recent molecular characterization studies indicate that there are many more species of *Cryptosporidium* than have previously been accepted (Fayer et al., 2001; Morgan-Ryan et al., 2002; Xiao et al., 2002). *Cryptosporidium canis* was proposed as the species infecting dogs (Fayer et al., 2001). This species had previously been referred to as the *C. parvum* dog genotype. *Cryptosporidium hominis* has been proposed as the species of *Cryptosporidium* infecting humans (Morgan et al., 2002). This species had previously been referred to as the *C. parvum* human or H genotype or genotype I. In the present study, molecular analyses at 3 loci have validated the species status of *C. galli*. At the 18S locus, *C. galli* isolates shared 96% similarity with *C. serpentis* and 95–96% similarity with *C. andersoni* and *C. muris*, respectively, and ~99% similarity with one another. The *C. galli* isolates shared 89–93% similarity with other *Cryptosporidium* species outside this cluster. At the HSP70 locus, *C. galli* isolates shared 94% similarity with *C. serpentis* and 93% similarity with *C. muris*. They shared 71–87% similarity with other *Cryptosporidium* species outside this cluster. At the actin locus, *C. galli* isolates shared 96% similarity with *C. serpentis* and 93% similarity with *C. muris* and *C. andersoni*. They shared only 76–84% similarity with other *Cryptosporidium* species. These molecular data, combined with morphological differences and biological differences, indicate that *C. galli* is a distinct species.

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