Cryptosporidiosis in the gourami *Trichogaster leeri*: description of a new species and a proposal for a new genus, *Piscicryptosporidium*, for species infecting fish

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ABSTRACT: *Piscicryptosporidium reichenbachklinkei* n. gen. n. sp. from the stomach mucosa of gourami *Trichogaster leeri* is described from ultrathin sections studied by electron microscopy. The new genus *Piscicryptosporidium* is proposed to include *P. reichenbachklinkei* n. sp., and *P. cichlidaris* n. sp., previously described as *Cryptosporidium* sp. from cichlid fishes of the genus *Oreochromis*. Another previously described *Chloromyxum*-like organism from *Sparus auratus* also appears to belong to this genus but fine structural data are not available for complete species diagnosis. All these 3 cryptosporidians are found in the stomachs of their piscine hosts and differ from all other known species of *Cryptosporidium* in that their sporulating oocysts sink into the basal part of the gut epithelium, or into the *Lamina propria*, rather than being evacuated into the gut lumen. In the species from gouramis and cichlids studied by electron microscopy, the microvilli of the epithelial host cells, or their vestiges, are retained on the surface of the parasitophorous sac enclosing the parasite.

KEY WORDS: *Piscicryptosporidium reichenbachklinkei* · Cryptosporidiosis · Stomach · *Trichogaster leeri*

INTRODUCTION

Landsberg & Paperna (1986) and later Paperna (1987), while describing fine structural affinities of cryptosporidians from cichlid fish, were aware of the unique nature of these organisms as compared with cryptosporidians from non-piscine hosts (O'Donoghue 1985, Fayer & Ungar 1986). However, they refrained from establishing a new genus on the basis of data from a single species. Recent recovery of cryptosporidians from reared gouramis *Trichogaster leeri* and the recognition of previously described *Chloromyxum*-like organisms infecting stomachs of juvenile *Sparus auratus* (Paperna 1983) as cryptosporidians have provided additional support for the segregation of piscine cryptosporidians into a distinct genus.

MATERIALS AND METHODS

Gouramis *Trichogaster leeri* were received from a commercial farm in Israel for disease diagnosis. The fish originated from a stock imported from an unidentified source in Southeast Asia. Direct examination revealed heavy cryptosporidial infection in the stomach mucosa. This was further confirmed with Giemsa-stained smears prepared from the stomach mucosa. Smears were air dried, fixed in absolute methanol and stained for 1 h in 10% Giemsa solution in pH 7.4 phosphate buffer. For transmission electron microscopy (TEM), tissue segments were fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) for 24 h at 4.0°C, rinsed repeatedly in the same buffer, post-fixed in 1.0% osmium tetroxide in the same buffer for 1 h and, after rinsing in the same buffer, dehydrated in graded alcohols and embedded in Agar 100 resin (Agar Co. UK). Thin sections, cut on a Reichert Ultracut

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ultratome with a diamond knife, were stained on grids with uranyl acetate and lead citrate and examined with a Jeol 100CX TEM.

Histological material [stained with hematoxylin-eosin, Periodic Acid-Schiff (PAS) and Best Carmine (BC) for glycogen; Paperna 1983] was photographed, and electron microscopic data from the first author's collection (see Landsberg & Paperna 1986, Paperna 1987) were used to provide amended descriptions of the previously reported species.

RESULTS

Piscicryptosporidium reichenbachklinkei n. gen. n. sp.

Host and locality: In the stomach of the gourami *Trichogaster leeri* reared in commercial-farm holding tanks in Israel (Lower Jordan Valley). Infection was recovered from 3 moribund specimens submitted for diagnosis.

Etymology: The new species is named in memory of the late Prof. Heinz-Hermann Reichenbach-Klinke, one of the great pioneers in the field of fish diseases (Hoffmann & Körting 1996), to acknowledge his important contribution to diagnosis and research of ornamental fish diseases.

Description (from TEM ultrathin sections). Endogenous stages were found within host-contributed parasitophorous sacs (PS) extending above the surface of the villous border of the stomach mucosal epithelium (Figs. 1 to 3). The PS retained short, widely spaced, rudimentary microvilli. The attachment zone contained the characteristic components described from cryptosporidians of cichlid fish (Landsberg & Paperna 1986), namely outer and inner electron-dense bands, each consisting of perpendicularly interwoven microfibrils (Figs. 8, 11 & 12). The junction zone with the host-cell cytoplasm contained numerous parallel microfibrils which joined perpendicularly to the outer band. The feeder organelle consisted of parallel array of very distinct endocytotic vesicles (Figs. 1, 2, 7 & 11). An annular ring formed an osmiophilic connection between the parasite cell wall and the boundary of the parasitophorous vacuole (PV) (Figs. 8, 11 & 12). Early attached trophozoites 1.3×0.5 to 2.5×0.1 μm were located within PS ranging in size from 1.5×0.9 to 2.7×1.6 μm (Figs. 1 & 10).

Premature meronts, $2.9\text{--}3.3 \times 2.3\text{--}2.6$ μm , were located in $3.2\text{--}4.2 \times 2.3\text{--}2.8$ μm PS. Their nucleus contained scattered chromatin of medium electron density. The cytoplasm was packed with ribosomes, and also contained intense network of rough endoplasmic reticulum (RE) (Figs. 2 & 3). In the dividing meronts, as well as in the developing merozoites, as in the premature meronts, the chromatin of the nucleus was scattered rather than aggregated. The cytoplasm in developing meronts and later in the meront's residuum retained the very intense network of ER, seen in early stages, whereas outlines of the other organelles and vesicles were faint (Figs. 3 & 9). Merozoite primordia were recognizable by their electron-dense rhoptry anlagen (Figs. 3 to 6). Divided meronts within a $2.7\text{--}3.4 \times 2.2\text{--}3.0$ μm PS contained up to 8 merozoites measuring $1.6\text{--}2.2 \times 0.45\text{--}1.4$ μm (Figs. 3 & 8). In the differentiated merozoites the rhoptries extended into their posterior end (Fig. 8). The meront's residuum retained its attachment zone with large, conspicuous feeder organelles (Figs. 7 & 8).

The only microgamont detected in the PS was 2.4×1.8 μm . Microgametes were characteristic of cryptosporidians, i.e. void of flagella (Fig. 10).

Macrogamonts, $2.8\text{--}3.3 \times 1.7\text{--}2.7$ μm in size, filled almost the entire PS space (Figs. 9 to 12). The nucleus contained a large central electron-dense nucleolus (Figs. 9 & 12); in older macrogamonts (zygotes?) the nucleoplasm became homogenous and diluted (Fig. 11). The cytoplasm contained extensive rough ER, a limited number of amylopectin granules, a variable number of vesicles filled with dilute, flocculent substance, one lipid vacuole and a body of irregular outlines containing electron-dense aggregate which could have been the wall-forming organelle (Figs. 11 & 12).

Early and sporulated oocysts, $2.4\text{--}3.18 \times 2.4\text{--}3.0$ μm (with mean diameter of 2.86 ± 0.29 μm), were bounded by a firm wall (which wrinkled following processing) and were located inside the mucosal tissue, enclosed within the remains of their PS (Fig. 13). At one end of the PS the remains of the attachment organelle were visible (Fig. 14). Oocysts contained up to 4 sporozoites; the latter, $3.6\text{--}4.0 \times 0.4\text{--}0.5$ μm , were densely filled with electron-dense substance containing variable-sized pale to white granule aggregates which seem to be crystalline bodies rather than assembled micronemes (Figs. 13 & 14). The host tissue at the perimeter of the oocyst was undergoing progressive degradation;

Figs. 1 to 16. Electron micrographs of *Piscicryptosporidium reichenbachklinkei* n. gen. n. sp. Abbreviations: a, amylopectin granules; ar, osmiophilic rings at the attachment zone; er, endoplasmic reticulum; ez, emerging merozoite; f, feeder organelle; ib, inner band and ub, outer band of the attachment zone; l, lipid vacuole; n, nucleus; ow, oocyst wall; ps, parasitophorous sac; r, rhoptry; ra, rhoptry anlagen; sa, sporozoite storage material granules; v, vesicles with flocculent contents; vi, microvilli; wf, the presumed wall-forming organelle; wg, white granules possibly a crystalline body; z, merozoites

Fig. 1. Early attached trophozoite. Fig. 2. Premature meront. Fig. 3. Early dividing meront (M) revealing both primordium of a merozoite and nucleus in the cytoplasmic residuum and a divided meront containing a formation of merozoites (dM) and a residuum (R). Fig. 4. Meront in early stage of division; merozoite primordia reveal rophtry anlagen. Figs. 5 & 6. Dividing meront, emerging merozoites reveal rophtries; the residuum is loaded with ER. Fig. 7. Divided meront, the residuum containing a large feeder organelle.

Fig. 8 Formation of merozoites still enclosed in the parasitophorous sac
 Fig. 9 Dividing meront (dM), with merozoites (z) and a residuum (R), young (jG) and differentiated macrogamont (G)
 Fig. 10 Young trophozoite (J) and a microgamont (Gi)
 Figs. 11 & 12 Magnified view of macrogamonts

Fig. 13. Oocyst inside the gastric mucosa. high magnification reveals the sporozoites (S) with the oocyst residuum filled with amylopectin granules Fig. 14. Oocyst released from the disintegrating gastric mucosa enclosed in its parasitophorous sac (PS) The PS retains remnants of the attachment organelle (ao) Figs. 15 & 16. Degraded gastric mucosa penetrated by many oocysts The oocysts within the tissue retain their parasitophorous sacs (PS)

oocysts were embedded inside a degraded cell matrix (Figs. 15 & 16), but around some, remnants of a PV were traced (Fig. 13). Under the increasing load of oocysts the host tissue eventually disaggregated and allowed the escape of some oocysts, with their envelopes, into the stomach lumen (Fig. 14).

Piscicryptosporidium cichlidis n. sp.

[= *Cryptosporidium* sp., of Landsberg & Paperna (1986) and Paperna (1987); Paperna et al. (1986), in an abstract of their communication to the Society of Proto-

zoologists, envisaging a forthcoming publication, referred to this species as *Cryptocystidium villithecum* Landsberg & Paperna. This name is, however, a *nomen nudum* since the intended publication was not realized.]

Host and locality (amended): Stomachs of cultured *Oreochromis niloticus* × *aureus* hybrid, fish ponds in the Beit Shaan Valley (Jordan basin), Israel (type host); *O. aureus*, Lake Kinnereth; *Tilapia zilli*, Lower Jordan valley, Israel.

Amended description. Meronts $2.1\text{--}3.7 \times 2.4\text{--}3.1 \mu\text{m}$ ($n = 6$), were enclosed in $2.1\text{--}3.9 \times 2.0\text{--}2.7 \mu\text{m}$ PS. Dividing meronts were enclosed in $3.3\text{--}3.9 \times 2.6\text{--}3.1 \mu\text{m}$ PS and yielded 4 to 8, $3.5 \times 0.6\text{--}0.9 \mu\text{m}$ ($n = 3$) mero-

zoites. Macrogamonts, $3.9\text{--}4.6 \times 2.2\text{--}3.4 \mu\text{m}$ ($n = 9$), were contained in $3.9\text{--}4.6 \times 2.3\text{--}3.6 \mu\text{m}$ PS. Microgamonts were enclosed in $3.1\text{--}4.4 \times 2.8\text{--}3.8 \mu\text{m}$ PS ($n = 2$). Sporulated oocysts, $4.0\text{--}4.7 \times 2.5\text{--}3.5 \mu\text{m}$ (mean $4.3 \pm 0.46 \times 3.25 \pm 0.64 \mu\text{m}$; $n = 5$), with up to 4 sporozoites, were contained inside a parasitophorous enclave within the gastric mucosa. Sporozoites $0.3\text{--}0.9 \times 3.6\text{--}4.1 \mu\text{m}$ ($n = 5$) contained crystalline bodies. In histological material examined by light microscopy, oocysts in the tissue seem to be intracellular.

Piscicryptosporidium sp.

Previously reported by Paperna (1983) as a *Chloromyxum*-like organism.

Host and locality: In the cardiac and pyloric regions of the gut of cultured *Sparus auratus*, Gulf of Aqaba, Red Sea.

Amended description. Stages presumed to be meronts and gamonts occurred on the mucous epithelium lining the gastric surface and the pits (but were absent from the fundic glands). PAS- and BC-negative stages with 1 or 2 nuclei were $2.9 \pm 0.4 \times 1.5 \pm 0.4 \mu\text{m}$ ($n = 18$). Spherical, $2.3 \pm 0.4 \mu\text{m}$ ($n = 5$) zoites contained 4 to 8 nuclei and several PAS- and BC-positive (diastase-resistant) vacuoles. Sporulating oocysts with 2 to 4 nuclei and many small PAS- and BC-positive vacuoles (apparently amylopectin granules), $3.1 \pm 0.5 \mu\text{m}$ ($n = 32$) occurred between the epithelial cells and in aggregates within oval enclaves in the basal portion of the epithelial layer (Fig. 17). The enclaves, $12.4 \pm 3.0 \times 7.4 \pm 1.3 \mu\text{m}$, were accompanied by a hypertrophic

nucleus, suggesting them to be enlarged host cells which had died following their invasion by oocysts.

Diagnosis of *Piscicryptosporidium* n. gen.

With the characters of the family Cryptosporidiidae, Leger, 1911 Oocysts with their parasitophorous sac sink to the basal portion of the gut epithelium, where they complete differentiation into sporozoites. Microvilli, or their vestiges, are retained on the surface of the parasitophorous sac.

Type species: *Piscicryptosporidium reichenbachklinkei* n. sp.

Other species: *Piscicryptosporidium cichlidis* n. sp. (= *Cryptosporidium* sp. of Landsberg & Paperna 1986).

Piscicryptosporidium sp. (= *Chloromyxum*-like organisms from *S. auratus*; Paperna 1983).

DISCUSSION

Piscicryptosporidium reichenbachklinkei conforms with *P. cichlidis* in most fine structural details, including both characters which have been proposed to be of generic significance, i.e. oocyst submerged in the mucosal tissue and microvillar rudiments retained on the PS. *P. reichenbachklinkei* TEM images of oocysts, as well as of most endogenous stages, are consistently smaller than those of *P. cichlidis* (2.86 ± 0.29 vs $4.3 \pm 0.46 \times 3.25 \pm 0.64$). Oocysts measured by LM from histological sections of *Piscicryptosporidium* sp. infected *S. auratus* stomach were $31 \pm 0.5 \mu\text{m}$.

The location of the oocyst in the tissue and the retained residual microvilli on the PS appear to be features unique to the piscine cryptosporidians, separating them from the species of *Cryptosporidium* parasitic in reptiles, avians and mammals. It has been found repeatedly in 3 piscine species and therefore provides a good argument for segregating the piscine cryptosporidians into a distinct taxonomic entity. The electron-microscopic images provided by Hoover et al. (1981) for *C. nasorum*, which was proposed by Levine (1984) to be the inclusive piscine species of *Cryptosporidium*, are too damaged to provide any conclusive structural data. Pavlasek's (1983) light microscopic report of an infection in carp does not provide information on the location of the oocysts.

Fine-structural differences among different species of *Cryptosporidium* are insignificant, even where experimental data and oocyst sizes suggest specific divergence (Fayer & Ungar 1986, Ostrovskaya & Paperna 1990). Nonetheless, current opin-

Fig. 17. Light microscopic view of histological section of *Sparus auratus* gastric mucosa infected by epiepithelial trophozoites (tz) and intraepithelial oocysts (oo) of *Piscicryptosporidium* sp.

ion is now deviating from Levine's (1984) methodology of assigning a universal species for each major vertebrate taxon. There is accumulating evidence for greater divergence among cryptosporidians occurring in hosts from the same major taxon: more than one species has been recognized in mammals and birds (Fayer & Ungar 1986) and there are hints of species divergence among *Cryptosporidium* infecting reptiles (Upton et al. 1989).

The structures identified as micronemes in sporozoites of *Cryptosporidium* spp. from *Agama stellio* (Ostrovská & Paperna 1990) and from snakes (Brownstein et al. 1977) seem to be crystalline bodies; these also seem to be characteristic of sporozoites of *Piscicryptosporidium*. In *P. reichenbachlinkei* most oocysts were embedded in degraded cell matrix, although around some, outlines of a PV were recognized. The intracellular location of *P. cichlidis* is certain (Landsberg & Paperna 1986). The accumulation of oocysts within the mucosal tissue and the consequent induced necrosis in the oocyst periphery seem to be the decisive factors in the pathological impact of this parasite's infection on fish: as infection intensifies and continues the accumulating oocysts are likely to induce extensive lesions in the stomach wall as evidenced by our histological and TEM images. This pathology is therefore expected to be different from that induced in infections of non-piscine hosts, where the entire endogenous cycle is 'epicytoplasmic'.

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