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Identification of New Morphological and Life-Cycle Stages of *Cochlosoma anatis* and Experimental Transmission Using Pseudocyst

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SUMMARY. *Cochlosoma anatis* is a flagellated intestinal parasite that infects a variety of avian species. *C. anatis* infections have been associated with decreased weight gain and increased morbidity and mortality. Conditions favoring the growth of this organism in birds are current pathogenic intestinal infections and/or young age. There is little data describing the life cycle of this parasite. In this study, electron microscopy images are presented that document longitudinal binary fission of the trophozoite stage and outline the events of pseudocyst formation, which includes a rounding stage. Evidence provided here indicates that the pseudocyst stage may be a mechanism for transmission of this organism. The observations reported here provide additional evidence of homology between *Cochlosoma* and members of the trichomonad order.

Key words: Cochlosoma, trophozoite, pseudocyst, poults, longitudinal binary fission, trichomonads

Abbreviations: SEM = scanning electron microscopy; TEM = transmission electron microscopy

*Cochlosoma anatis* is a protozoal organism found in various avian species. Kotlan first described *C. anatis* from the intestinal contents of European domestic ducks in 1923, which was later reviewed by Travis (13,25). The trophozoite stage of this parasite is ovoid to pyriform in shape and 6–12 μm long by 4–7 μm wide with six flagella, an axostyle, lateral groove, and undulating membrane (2,15). Trophozoites are uninucleate and have a prominent ventral adhesive disk (2,6). The disk serves to adhere the parasite to the intestinal mucosa of the host (6,21,29). Attachment to the microvillus border of the intestines appears to be the main host–parasite interaction; however, whether pathogenicity is due to destruction of the microvilli or mechanical blockage of nutrient absorption is unclear (2,6,21,29).

*C. anatis* species have been observed to infect turkeys, chickens, bobwhite quail, songbirds, waterfowl, bats, and shrews (2,4,5,6,7,14,15,20). The trophozoites may infect the whole intestinal track but can also be restricted to one or several locations (4,6,15,20,25,29). Oral transmission has been demonstrated for turkeys, chickens, bobwhite quails, and ducks. Trophozoites are shed in fecal material at approximately 5–7 days postinoculation (4,15). Infected turkeys are depressed, have ruffled feathers, and may have diarrhea resulting in fluid-filled, swollen small intestines and ceca, often observed during necropsy (2,6,19).

*C. anatis* infections have been linked to running of ducklings, enteritis in turkeys, and mortality, dehydration, and malabsorption of young finches (4,6,16,22). Cooper et al. reported that a 16% decrease in body weight was associated with *C. anatis* infection in turkeys (6). Reduced fat absorption has been observed in turkey poults infected with *C. anatis*, but final weight and performance parameters were not affected (3). *Cochlosoma* is often present with other intestinal pathogens, and coinfection of *C. anatis* and turkey coronavirus has been shown to be more pathogenic for infected turkeys than an infection with either pathogen alone (24). There is no known procedure for purifying or culturing this parasite, which makes studying *C. anatis* difficult (2,27). Advantageous conditions for *C. anatis* are current pathogenic intestinal infections or young age.

Morphologic similarities between *Giardia, Trichomonas,* and *Cochlosoma* have been well documented (6,20,21). The adhesive disk is the main characteristic that *Giardia* and *Cochlosoma* share (2,14); however, Pecka et al. demonstrated ultrastructural differences in the adhesive disk of these species and concluded that, based on detailed ultrastructural analysis, *Cochlosoma* has a closer relationship with trichomonads (21). Both *Cochlosoma* and *Trichomonas* are uninucleate and have a parabasal apparatus, a tubular axostyle, and a crescent-shaped pelta (14). Recently, Vrlik et al. confirmed, by sequence analysis of the 16S rRNA gene, that *Cochlosoma* is genetically similar to *Trichomonas* (26).

*C. anatis* has been observed to divide by longitudinal binary fission of the trophozoite, which is consistent with most species from the class Zoomastigophorea (2,25). Kotlan observed longitudinal division and a cyst stage, but did not publish drawings or dimensions, and there are no other detailed descriptions of a *C. anatis* cyst or pseudocyst stage (2,13,25). This article presents evidence...
supporting longitudinal binary fission of the trophozoite and demonstrates pseudocyst formation of *C. anatis*.

**MATERIALS AND METHODS**

Several poultry farms with a history of *C. anatis* infections were identified for sampling. Turkey poults were euthanatized and intestinal scrapings from the area around Meckel diverticulum were collected. These scrapings were mounted on slides and examined with light microscopy for the presence of *C. anatis* and the absence of other protozoa (11). Samples containing *C. anatis* were diluted in saline solution, then stored on ice for 24 hr and re-examined by light microscopy. No trophozoites were observed, and only pseudocyst-like stages were noted. Using this isolate, a 1-day-old poult was gavaged with 1 ml of the sample and then housed with five other poults of the same age. After 48 hr, all six of the turkeys were necropsied and sampled as previously described. Chilled, preservative-free saline, containing boric acid, was added to the sample at a 1:1 ratio. The sample was then left at room temperature for approximately 20 min and then fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer.

**Scanning electron microscopy (SEM).** Approximately 0.5 ml suspension of the fixed *C. anatis*-containing sample was transferred by positive pressure to a 0.2 μm Nuclepore polycarbonate filter membrane using a 1 mm³ syringe. Polycarbonate filters with attached cells were postfixed in 1% osmium tetroxide in 0.1 M sodium phosphate buffer for 1 hr, washed in 0.1 M phosphate buffer, and dehydrated through a series of ethanol solutions. The filters were critical-point dried, mounted on aluminum stubs, coated with gold, and examined with a Phillips 505 scanning electron microscope operating at 15 kV.

**Transmission electron microscopy (TEM).** A fixed suspension containing *C. anatis* was pelleted in 2% agar by centrifugation, washed with 0.1 M sodium phosphate buffer, postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, and dehydrated through a series of ethanol solutions. The samples were embedded into Polybed 812 and cured for 48 hr. Ultrathin sections were stained with 2% uranyl acetate and Reynold lead citrate and examined with a Zeiss 10 CA transmission electron microscope.

**RESULTS**

After storage on ice for 24 hr, no trophozoites were seen when examined by light microscopy; however, pseudocyst-like stages were observed. Electron microscopy observations suggest that the flagella and other external structures were internalized and the observed organisms were lacking a true cyst wall. Infected poults housed with noninfected hatch mates for a 48-hr incubation period resulted in all of the turkeys acquiring *C. anatis* infections.

**Scanning electron microscopy.** The trophozoites of *C. anatis* were determined to be 6–12 μm long by 4–7 μm wide and showed a prominent adhesive disc at the anterior end of the pyriform body (Fig. 1A). A thin, pointed axostyle projected from the posterior end of the body, and a lateral groove was observed to extend from one side of the adhesive disc to the posterior end of the parasite. The four anterior flagella and the recurrent flagellum appeared to emerge from the adhesive disc just above the origin of the lateral groove. The undulating membrane ran along the lateral groove the entire length of the body. A sixth flagellum that was attached to the dorsal surface was not shown here (21).

Observations showed that the rounding stage of *C. anatis* was 5–9 μm in diameter and maintained the adhesive disc (Fig. 1B). The thin, pointed axostyle was no longer visible at the posterior end of the body, and the flagella appeared to be undergoing internalization. The shape of the parasite was no longer pyriform, but was rounded with a concave or flattened area where the adhesive disc was located on the cell surface. The undulating membrane and lateral groove were less apparent during this stage.

The pseudocyst stage of *C. anatis* was observed to be 5–9 μm in diameter, slightly ovoid in shape, and exhibited a small protuberant structure (Fig. 1C). The flagella and adhesive disc were no longer visible on the outer surface of the cell, and the lateral groove and undulating membrane were no longer apparent.

**Transmission electron microscopy.** Longitudinal sections from the trophozoites of *C. anatis* showed an adhesive disc skeleton...
The kinetosomal complex of the four anterior flagella were plainly visible, as was the striated costa, which began at the base of the kinetosomes, continued to the posterior end of the parasite, and was branched into two sections. The lamellar branch supported the lateral groove, and the fibrillar branch followed the undulating membrane. The recurrent flagellum was perpendicular to the kinetosomes of the anterior flagella and was adhered to the undulating membrane, which continued posteriorly. The Golgi complex was located on the dorsal side just below the dorsal flagella, while the nucleus was located in the anterior of the cell and was surrounded by a double membrane. Double membrane, hydrogenosome-like organelles were visible in the cytoplasm along with vacuoles, while mitochondria were absent. Evidence of the axostyle, axostylar junction, and the parabasal fiber could be seen.

Cross sections of the anterior section of the trophozoite showed that the lateral groove was divided into two sections by the undulating membrane (Fig. 2B,C). One section contained the four anterior flagella, and the other contained the recurrent flagella. The Golgi complex lay in close proximity to the parabasal fiber, which was attached to a kinetosome. Striated lamella fibers were attached to the lamellar branch of the costa, and the fibrillar branch of the costa was also readily visible (21). Cross sections of longitudinally dividing trophozoites were observed and revealed two sets of Golgi complexes, parabasal fibers, undulating membranes, lamellar and fibrillar branches of the costa, recurrent and anterior flagella, and nucleiuses (Fig. 2D,E).

Examination of the rounding stage showed that the adhesive disc skeleton and costa apparently migrated from the cell surface to the interior of the cell (Fig. 3A,B). Extracellular flagella were still noted, and the prominent nucleus, hydrogenosome-like organelles, and vacuoles were readily visible. The cytoplasm of the rounding cell stained a lighter shade, signifying that the protein content of the cytoplasm had changed. The undulating membrane and lateral groove were not seen during this stage.

Cross sections of the pseudocyst stage indicated that the anterior flagella were internalized (Fig. 4A,B). The kinetosomal complex, adhesive disc skeleton, and other support structures remained intact and appeared to be compartmentalized to one section of the cell. Evidence of the recurrent flagella was sometimes seen outside of the cell. The nucleus, hydrogenosome-like organelles, and vacuoles could be seen in the cytoplasm during this stage. The cytoplasm of the pseudocyst had returned to the same shade as the cytoplasm of the trophozoites. The undulating membrane and lateral groove were not seen. The pseudocyst stage appeared to be lacking a true cyst wall.

**DISCUSSION**

After a 48-hr incubation period, a turkey poult gavaged with a pseudocyst-containing sample developed an active *C. anatis* infection. This demonstrates that the pseudocyst form of *C. anatis* may be capable of producing an infection when contracted orally by young pouls. Five birds housed with an experimentally infected bird all became infected with *C. anatis*. This data suggests that organisms shed in the fecal material of pouls infected with the pseudocyst stage are capable of infecting other pouls.

SEM observations of trophozoites are consistent with published reports on the external morphology of *C. anatis* (2,6,15,21). The images show that there is a distinct intermediate stage between the trophozoite and pseudocyst stage. The rounding stage serves as this intermediate stage, in which external structures are internalized inside the cell. The adhesive disc and axostyle tip change shape, and the cell becomes more circular. The images indicate that the flagella were internalized starting from the base and working toward the tip. The undulating membrane appears to be internalized as well. The pseudocyst stage had no external structures, but a small structure protruded from the side of the otherwise round cell membrane.

TEM observations of trophozoites presented here confirm observations by Pecka et al. in 1996 (21). Trophozoites have been reported to divide by longitudinal binary fission, but little evidence was provided to support this theory (2,13,21,25). Images collected in this study support the theory and provide evidence that the trophozoites replicate and divide by longitudinal binary fission. Organelles and support structures are divided among the daughter cells evenly, and nuclear material contained in the nucleus appears to have replicated and dispersed to individual nuclei during this process.

TEM observations of the rounding stage support the theory that this is an intermediate stage in which external structures are internalized and compartmentalized inside the cell. The cytoplasm stains a lighter shade during this stage; the cause of this is still unknown. Organelles, such as vacuoles, the hydrogenosome-like organelles, nucleus, costa, and the adhesive disc, which are found in the trophozoite, can be found in the rounding stage. The undulating membrane and lateral groove are not seen during this stage. The adhesive disc and costa appear to be relocating from the exterior of the cell and condensing in one central location on the interior of the cell.

Examination of the pseudocyst stage showed that the costa, adhesive disc, and anterior flagella were internalized and compartmentalized in the cytoplasm. The nucleus, hydrogenosome-like organelles, and vacuoles were still visible in the cytoplasm during this stage. The structures all appeared to be intact; however, the undulating membrane and lateral groove were not seen. The absence of these structures on the surface of the cell reduces the surface area, effectively reducing exposure to harmful environmental factors (9). Evidence of the recurrent flagellum was often seen on the exterior of the cell. This may indicate that the recurrent flagellum is slow to be internalized. Granger et al. reported similar findings for *Trichomonas fetus*. When the trophozoite form of the cells were cooled to 0 C and then left at room temperature, the anterior flagella were internalized more rapidly than the recurrent flagella (9). This newly described stage of *C. anatis* is lacking a true cyst wall and is best classified as a pseudocyst.
The process of pseudocyst formation of *C. anatis* may be a reversible event, taking place with or without cell division. Granger et al. presented data showing that trophozoites of *T. fetus* underwent pseudocyst formation when the cells were cooled to 0°C for 60 min and then rewarmed to 37°C. *T. fetus* cells internalized their flagella within 3 min and then gradually externalized them, and TEM data showed the pseudocyst stage of *T. fetus* dividing after a similar treatment (9). Pseudocyst formation, which refers to compact, nonmotile forms without a true cyst wall, has also been described for many other gastrointestinal trichomonads (1,8,9,10,12,17,18,23,28). As noted, *C. anatis* has been reported to be closely related to trichomonads and may undergo similar pseudocyst formation (6,14,20,21,26). Pseudocyst formation is most likely a self-preservation mechanism that allows *C. anatis* to survive subphysiological temperatures and/or other adverse environmental conditions, until a suitable host ingests them. Vulnerable structures like the adhesive disc and the undulating membrane are internalized to decrease the surface area that is exposed to environmental stress. The flagella also

Fig. 3. Transmission electron micrographs of *C. anatis* rounding stages. (A) Longitudinal section of a trophozoite next to a section of a rounding stage shows the change in cytoplasm staining. Adhesive disc (ad), costa (c), and nucleus (n). Bar = 2 μm. (B) Section of a rounding stage shows the migration of the adhesive disc (ad) and the costa (c) to the interior of the cell. Hydrogenosome-like organelles (h). Bar = 500 nm.

Fig. 4. Transmission electron micrographs of *C. anatis* pseudocysts. (A) Section of a pseudocyst. Kinetosomes (k), costa (c), and nucleus (n). Bar = 2 μm. (B) Section of a pseudocyst. Nucleus (n), hydrogenosome-like organelles (h), and a compartmentalized protuberance in the cell membrane containing cytoplasm, kinetosomes, adhesive disc skeleton, and other support structures (arrowhead). Bar = 2 μm.
appear to undergo internalization, but more data are required to confirm this process. Because these structures are attached or continuous with the plasma membrane, damage to one of these structures would be detrimental to the cell (1). Further studies are necessary to understand the mechanism by which C. anatis trophozoites undergo longitudinal binary fission and pseudocyst formation and what action can be taken to control the infectivity of this organism.

REFERENCES


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