Three-Dimensional Imaging of Monogenoidean Sclerites by Laser Scanning Confocal Fluorescence Microscopy

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RESEARCH NOTES

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Prevalence of *Toxoplasma gondii* Antibodies in Rabbits (*Oryctolagus cuniculus*) From Mexico

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ABSTRACT: Antibodies to *Toxoplasma gondii* were determined by indirect enzyme-linked immunosorbent assay in serum samples from domestic rabbits from 3 rabbit farms in Mexico. Antibodies to *T. gondii* were found in 77 (26.9%) of 286 animals. On the farm with the higher rearing standards, the seroprevalence was 18.7%, whereas on the farm with medium standards and another managed by a family, seroprevalence was 39.7 and 33.3%, respectively. This report is the first report concerning the prevalence of antibodies to *T. gondii* in rabbits from Mexico. Although the prevalence found in the present study is within the range reported for other countries, 2 of the farms revealed a relatively high prevalence, which was probably associated with the presence of cats inside rabbit houses.

The apicomplexan parasite *Toxoplasma gondii* is unusual in its ability to infect a wide range of vertebrate intermediate hosts. Infection generally occurs either through ingestion of oocysts shed in the feces of a cat, the definitive host, or of viable tissue cysts consumed in raw or undercooked meat (Levine, 1973).

Toxoplasma gondii is prevalent in many of warm-blooded animal species, including humans (Dubey and Beattie, 1988). Cysts are more frequently observed in tissues of pigs, sheep, and goats, but they also can be found in other commercially raised animals, such as chickens, horses, and rabbits (Tenter et al., 2000; Russell et al., 2002). In the latter mammals, the seroprevalence ranges from 2 to 53% in countries such as China, the Czech Republic, Germany, Egypt, France, and United States. There have been several reports of toxoplasmosis outbreaks in wild and domestic rabbits around the world (Dubey et al., 1992; Haziroglu et al., 2003; Almería et al., 2004).

Although *T. gondii* is not a significant problem for healthy individuals, the proliferative phase (tachyzoite) may be clinically important, especially in pregnant and suckling rabbits. When it is transmitted through the placenta, it is highly pathogenic for the embryos (Leland et al., 1992; Burato and Brusia, 1995).

In domestic rabbits, *T. gondii* infection also has implications for public health, because the tissue cyst can be transmitted by meat consumption to the definitive host and to other intermediate hosts, including humans (Dubey and Beattie, 1988; Sroka et al., 2003; Almería et al., 2004).

The present study reports the prevalence of *T. gondii* antibodies in domestic rabbits (*Oryctolagus cuniculus*) on 3 rabbit farms in central Mexico. The first farm is a commercial farm associated with the facilities of the University of Chapingo, State of Mexico (approximately 60 km from Mexico City). It has an intensive production system and high safety measures. This place was considered of low risk for *T. gondii* transmission. The second farm is a teaching farm, located at the Faculty of Veterinary Medicine of the National Autonomous University (UNAM) in Mexico City. This farm has a semi-intensive production system and medium-level safety measures. Finally, the third farm is managed by a local family. It is situated in the State of Puebla (150 km from Mexico City) and had very low standards of equipment, management, and sanitary measures. At each rabbit farm, rearing conditions were investigated through a questionnaire submitted to the managers, and, when possible, by direct observation.

Blood samples were carefully taken from the auricular marginal vein of 286 adult rabbits (farm I, n = 160; farm II, n = 78; and farm III, n = 48). At the laboratory of the Parasitology Department of the Faculty of Veterinary Medicine of UNAM, the samples were centrifuged to separate the serum, which was stored at −20 C until analysis by indirect enzyme-linked immunosorbent assay (ELISA) to test for IgG antibodies to *T. gondii*.

The tachyzoites of the RH strain of *T. gondii*, maintained by repeated passages in the peritoneal cavity of Balb/c mice, were harvested in 0.01 M phosphate-buffered 0.15 M saline, pH 7.2 (PBS). Contaminating mouse cells were removed by centrifugation at 1,200 rpm for 5 min. Tachyzoites >95% pure were sonicated for 30 sec 7 times on ice, in an ultrasonic homogenizer (Cole Parmer, Vernon Hills, Illinois). The mixture was centrifuged at 30,000 rpm for 60 min at 4 C in a Beckman XL-90 centrifuge, with a SW50.1 rotor. The protein concentration of the supernatant was determined following the method of Bradford with the Bio-Rad (Hercules, California) reagent (Bradford, 1976); then, it was fractioned and stored at −70 C until use.

To obtain positive and negative control samples, 3 rabbits that were raised in the animal house of the National Institute of Pediatrics of the Ministry of Health (INPed) and that tested negative by Western blot (Vela-Amieva et al., 2005) were experimentally infected with 100,000 tachyzoites of the RH strain of *T. gondii*, and maintained with purified water and high-standard commercial food for up to 2 mo. Using pre- and postinfection samples, we determined optimal concentration of antigen and dilutions of the serum samples and of the goat anti-rabbit IgG–horseradish peroxidase conjugate.

The ELISA was performed as follows. The plates (Maxisorp, Nalgene Nunc, Rochester, New York) were coated with 100 μl/well of *T. gondii* antigen at a concentration of 4.5 μg/ml diluted in 0.1 M carbonate buffer, pH 9.6, overnight at 4 C, and then washed 3 times with 200 μl/well of PBS with 0.05% Tween 20 (PBS-T). After blocking with 1% bovine serum albumin in PBS-T for 30 min at 37 C, washes were repeated with PBS-T 3 times and with PBS 2 times. The serum samples, diluted 1:200 in PBS-T, were incubated for 2 hr at 37 C, and the plates washed as described above. The conjugate was used at a dilution of 1:500 in PBS-T and incubated for 2 hr at 37 C. After further washing with PBS-T and PBS, the substrate–chromogen solution (4 mg of orthophenylenediamine in 10 ml of 0.1 M citrate-citric acid and 4 μl of 30% H_2O_2) was added and incubated until color development. The reaction was stopped with 50 μl/well of 1 N sulfuric acid, and the absorbance was registered at 492 nm. The serum samples from 7 rabbits raised at the INPed animal house and negative by Western blot were processed. The mean plus 3 SDs of these samples was taken as the cut-off point for the samples taken from the farms.

The statistical analysis was performed using the Epi-INFO 2002 software of the Centers for Disease Control and Prevention (Atlanta, Georgia). Comparison of prevalence between farms was performed by chi-square test.

Antibodies to *T. gondii* were found in 77 (26.9%) of 286 rabbits. In the farm with better conditions, the seroprevalence was 18.7%, whereas in that with medium standards and the one managed by the family, it was 39.7 and 33.3%, respectively. The difference in prevalence between the first farm and the other 2 farms was statistically significant ($P < 0.001$).

Table I shows the rearing conditions and some risk factors that influence the presence of *T. gondii*. In all farms, the rabbits were kept in cages and fed ad libitum with commercial pellets. The presence of cats in rabbit houses, bedding material of the nest boxes, and safety measures varied among farms. The farm in Puebla presented more risk factors than the other 2 facilities.

In the present study, we observed different prevalences of *T. gondii* antibodies between a well-controlled rabbit farm and other 2 farms with less careful management. Because rabbits are herbivorous, the more plausible source of infection was contamination of food or water with oocysts, which may be disseminated through wind, rain, or stored foods. Invertebrates such as earthworms, flies, and cockroaches also have been postulated as transport hosts (Frenkel et al., 1970). In this regard, we

TABLE I. Risk factors searched in the 3 farms studied.

Farm	Chapingo	University (UNAM)	Puebla
Rabbit houses	Yes	Yes	No
Cats within rabbit house	No	Yes	Yes
Noxious fauna	Flies	Flies, births	Flies, births
Bedding material of the nest box	Sawdust	Sawdust, hay	Straw, hay
Type of food	Commercial pellets	Commercial pellets	Commercial pellets and fresh alfalfa
Safety measures	Foot baths, restrict visitors	Foot baths, restrict visitors	None
Carcass disposal	Buried or incinerated	Buried or incinerated	Buried
Sealed water deposit	Yes	Yes	Yes

observed young cats inside the rabbit houses and the food stores both in the University and Puebla farms, and even above cages in the farm of Puebla.

Hay, straw, and grain that had been contaminated with cat feces have been identified as sources of infection for livestock (Tenter et al., 2000). In the present case, the bedding material (sawdust, straw, hay, or alfalfa) may be contaminated with oocysts, thereby infecting rabbits.

In the Chapingo farm, the prevalence was lower and may be because of better measures of hygiene and confinement; moreover, cats were not observed within the facility. We cannot rule out the possibility that positive rabbits became infected before introducing them to the farm, because on occasion this farm buys rabbits from other sources. Moreover, congenital transmission could not be ruled out as a route of transmission.

The role of domestic rabbits in the epidemiology of toxoplasmosis has not been established, but it is a potential source of infection to humans and other species (Sroka et al., 2003). To prevent the transmission of *T. gondii*, the cats should be kept away from the rabbit houses, food stores, and water storage facilities.

This report is the first report concerning the prevalence of antibodies to *T. gondii* in rabbits from Mexico. Although the prevalence found in the present study is within the range reported for other countries, 2 of the farms presented a relatively high prevalence. Rabbit meat is not preferred by the Mexican people, but it is consumed, especially in certain areas of the country. Thus, these results could have public health implications.

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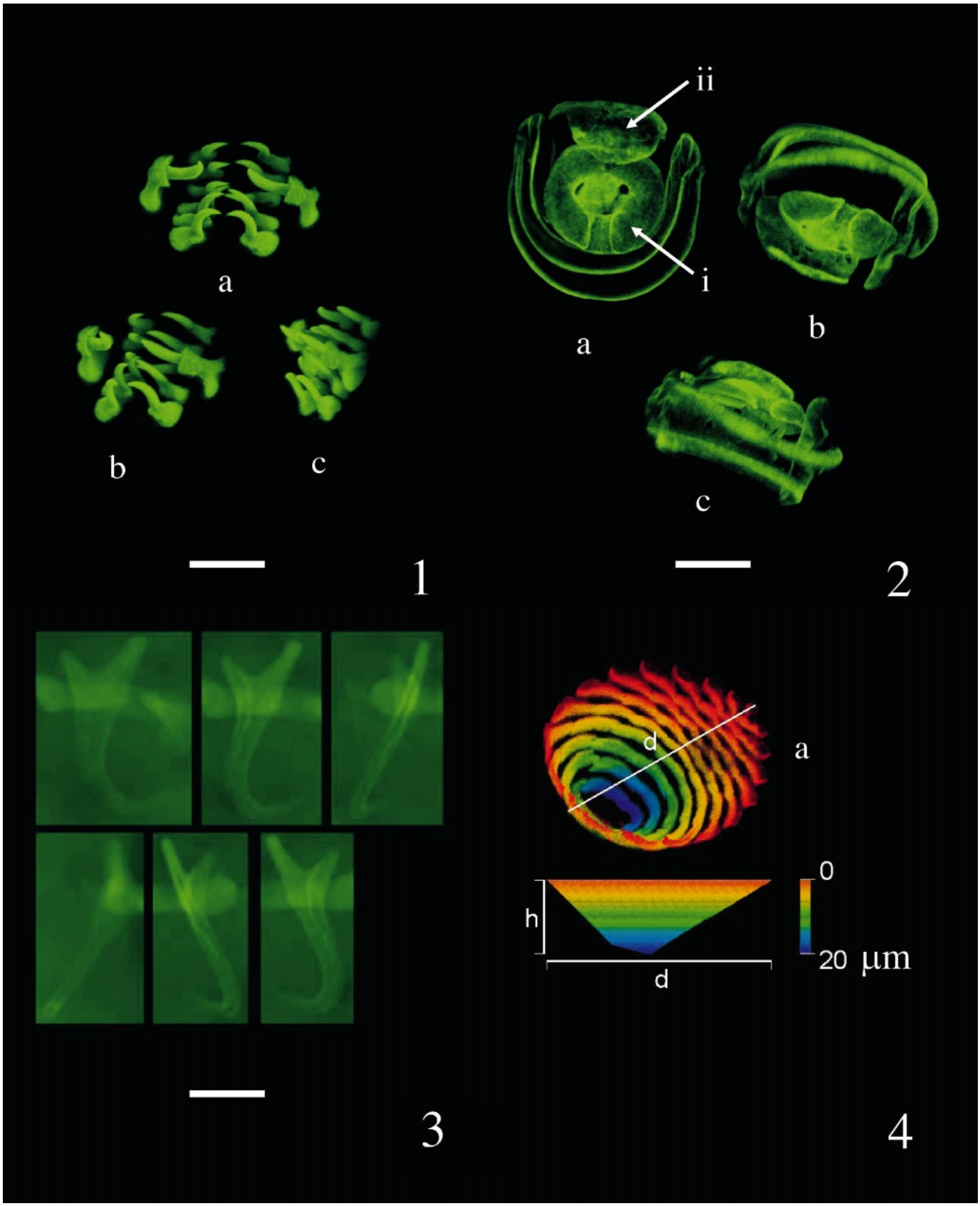
ABSTRACT: A nondestructive protocol for preparing specimens of Monogenoidea for both α-taxonomic studies and reconstruction of 3-dimensional structure is presented. Gomori's trichrome, a stain commonly used to prepare whole-mount specimens of monogenoids for taxonomic purposes, is used to provide fluorescence of genital spines, the copulatory organ, accessory piece, squamodisc, anchors, hooks, bars, and clamps under laser scanning confocal microscopy.

The copulatory and haptoral sclerites of monogenoids are microscopic and intricately complex. They vary between closely related species more than many other anatomical structures and thus provide important characters for species identification, diagnosis of higher taxa, and for phylogenetic and coevolutionary studies (Gusev, 1978; Boeger and Kritsky, 1989, 1997, 2001; Gerasev, 1989, 1998; Desdevises et al., 2001; Kritsky and Boeger, 2003). Traditional methods for visualizing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and recording morphological features of haptoral and copulatory sclerites involve light microscopy (bright field, phase contrast, or Nomarski interference) followed by hand illustration by using a camera lucida or microprojector, but ensuing representations rarely convey the 3-dimensional aspect of the structures. The limited depth-of-field of light transmission instruments generally requires flattening of specimens, often resulting in distorted or damaged sclerites, to visualize a 2-dimensional aspect (see methods introduced by Malmberg, 1957; Ergens, 1969; Žd'árská, 1976; Kritsky et al., 1978). Scanning (SEM) and transmission (TEM) electron microscopy also have been used to determine morphology and structure of these sclerites (Justine, 1993; Shinn et al., 1993). Although some of these sclerites may be released from overlying tissue with digestion or sonication techniques without significant damage for SEM (Shinn et al., 1993), use of SEM and TEM requires destruction of the helminth specimen.

Laser scanning confocal fluorescence microscopy (LSCFM) provides a means of determining 3-dimensional structure without destruction or damage of the biological specimen. For example, Zill et al. (2000), Klaus et al. (2003), and Schawaroch et al. (2005) reconstructed the 3-dimensional aspects of insects' exoskeleton and genitalia with confocal imaging of endogenous fluorescence. Neves et al. (2001) and Neves et al. (2005) used the technology to compare morphological changes in the tegument of the trematode *Schistosoma mansoni* and to describe the reproductive system of stained specimens of this species. Studies incorporating confocal microscopy using immunocytochemistry applied to neurobiology of parasitic flatworms, including monogenoids, are not recent (Halton et al., 1992) and have been expanded to include studies on other physiological processes exhibited by monogenoids (i.e., Schabussova et al., 2003, 2004). However, minimal use of the technology has been applied to the morphological characterization of the sclerites of these flatworms. Autofluorescence apparently does not occur in these sclerites, and previous effort to use the technology on stained specimens has produced marginal results (Kohn et al., 2000). The aim of the present article is to report on a methodology developed to obtain 3-dimensional imaging of monogenoidean sclerites by LSCFM from specimens prepared for α-taxonomy and suitable for serving as reference or type material.

Specimens of various species of marine and freshwater monogenoids fixed and mounted in different fixatives and mountants were used in development of an effective protocol for obtaining 3-dimensional reconstructions of sclerites by using LSCFM (Table I). LSCFM images were collected using the Leica TCS SP2 confocal microscope equipped with an inverted Leica DMIRE2 microscope and a PL APO ×63 oil immersion objective (numerical aperture = 1.4). A krypton-argon laser was used for fluorescence excitation at 515 nm; fluorescence emission was detected between 525 and 730 nm. Images were collected at 8-bit with 1,024 by 1,024 pixels per frame. The width of the scanned field varied between 20 and 70 μm. Tomographic series for 3-dimensional reconstructions were collected using a step size of 0.115 μm, which provided maximal resolution along the z-axis. Three-dimensional reconstructions, the projections of the image stacks, and color-coded topographic images were obtained using Leica LCS software.

No fluorescence was observed from fresh specimens mounted on a slide in a drop of tap water or in ammonium picrate glycerin. Specimens fixed with alcohol-formalin-acetic acid (AFA) and subsequently mounted in glycerin gelatin also did not demonstrate fluorescence. However, haptoral and copulatory sclerites of specimens fixed in AFA or hot 5% (60 C) formalin, stained with Gomori's trichrome, and mounted in Canada balsam did fluorescence if excited at 515 nm by the krypton-argon laser. Marine parasites showed a higher level of fluorescence than those from freshwater, but this difference disappeared when specimens were exposed to 1 N NaOH before staining. Although no significant difference in fluorescence was detected using AFA or hot formalin as fixative, hot formalin provided relaxed specimens, whereas those fixed in AFA usually were contracted and were less desirable for study of other morphological features of the helminth by using light microscopy.

All copulatory and haptoral sclerites, including genital spines, the copulatory organ, accessory piece, squamodiscs, anchors, hooks, bars, and clamps, demonstrated fluorescence when stained with Gomori's trichrome. Figure 1 shows a 3-dimensional reconstruction of the genital corona of *Kuhnia scombri* (Heteronchoinea, Mazocraeidae) obtained through LSCFM; the genital corona is rotated to show the structure in dorsoventral view (Fig. 1a), diagonal view (Fig. 1b), and lateral view (Fig. 1c). Figure 2 demonstrates rotation of a haptoral clamp of *K. scombri* through 3 views (Fig. 2a, lateral view; Fig. 2b, diagonal view; and Fig. 2c, dorsoventral view). Figure 3 shows 6 perspectives of the ventral anchor of *Pseudorhabdosynochus* sp. (Polyonchoinea, Diplectanidae) rotated through approximately 180° (the horizontal structure in this figure represents a portion of the dorsal bar).

Based on comparative results, the following method of collection and preparation of helminths is recommended for obtaining specimens for both 3-dimensional visualization and provision of appropriate material for α-taxonomic purposes. Gill baskets of respective hosts, removed at the site of collection, or the entire host, if small, are placed in containers of hot 5% formalin (60 C) to relax and fix the attached monogenoids. Fixed gills are placed in vials containing the respective fixative, labeled, and stored until ready for study. A formalin-fixed specimen(s) is subsequently removed from the gills or picked from the sediment by using a fine probe and dissecting microscope. The specimen is placed in a droplet of Gomori's trichrome stain (Humason, 1979) located near the center of a small disposable Petri dish. After 1–2 min, the droplet containing the specimen(s) is flooded with absolute ethanol to cease the absorption of stain. Destaining of the specimen(s) is accomplished by adding water to the dish to dilute the ethanol–stain mixture to about 50%. When the desired level of stain remains in the specimen, the specimen is removed with a fine probe and placed in absolute ethanol for about 1 min, after which the specimen is transferred to beachwood creosote for clearing. The specimen is subsequently mounted in Canada balsam. This procedure furnishes relaxed and well-preserved specimens for taxonomic purposes and with sclerites that fluoresce under confocal microscopy. Fluorescence may be enhanced somewhat by placing the formalin-fixed specimen in 1 N NaOH for 10 min before staining.

An added application of LSCFM by using the Leica LCS software is the demonstration of 3-dimensional perspectives by using color coding of the image stack. Although usually depicted as a flat 2-dimensional surface in traditional drawings, the squamodiscs of diplectanids are cone-shaped, which apparently allows development of differential pressures during attachment of the parasite to the host's skin or gill surfaces. Figure 4 shows a 3-dimensional representation of the squamodisc of *Pseudorhabdosynochus* sp., which is color coded to indicate different levels or depth of the structure. The use of different colors, each corresponding to a different level of depth, allows a clearer understanding of the morphology of the attachment structure. For example, the squamodisc of *Pseudorhabdosynochus* sp. illustrated in Figure 4 is clearly cone shaped, with an estimated depth of 20 μm.

Not only does LSCFM provide a distinct advantage over traditional methods of visualization by providing improved quality of information concerning the morphology of monogenoidean sclerites but also it allows the determination of the morphology of sclerites of unflattened specimens whose sclerites are not situated along the same plane as that of the microscope slide. Many reference and type specimens of monogenoids deposited in museums are stained with Gomori's trichrome since introduction of the stain by Kritsky et al. (1978). It is now possible to review this material to obtain additional morphological information

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FIGURES 1–4. Confocal images of copulatory and haptoral sclerites of monogenoids. (**1**) Three-dimensional reconstruction of the genital corona of a specimen of *K. scombri* rotated through 3 views (a. Dorsoventral view. b. Diagonal view. c. Lateral view). Bar = 20 μm. (**2**) Three-dimensional reconstruction of a clamp of *K. scombri* rotated through 3 views (a. Lateral view. b. Diagonal view. c. Dorsoventral view. i. Posterior midsclerite. ii. Anterior midsclerite). Scale bar = 20 μm. (**3**) Six perspectives of the ventral anchor of *Pseudorhabdosynochus* sp. showing rotation of the anchor through approximately 180° (the horizontal structure in this figure represents a portion of the dorsal bar). Bar = 20 μm. (**4**) Color-coded projection of the 3-dimensional reconstruction (**a**) and topographical profile of the ventral squamodisc of *Pseudorhabdosynochus* sp. along its diameter (**d**). Colorimetric-scale depth (**h**) increasing from orange (surface) to blue (innermost).

TABLE I. Parasite species, their hosts, and sample preparations used for development of a protocol for 3-dimensional reconstruction of monogenoidenan sclerites by using LSCFM.

Parasite	Host	Sample preparation	Fluorescence
Dactylogyrus extensus	*Cyprinus carpio*	Fresh specimen on slide in tap water	−
Dactylogyrus extensus	*Cyprinus carpio*	Fresh specimen on slide in ammonium picrate glycerin	−
Dactylogyrus extensus	*Cyprinus carpio*	Specimen fixed in AFA, mounted in glycerin gelatin	−
Dactylogyrus extensus	*Cyprinus carpio*	Specimen fixed in hot 5% formalin (60 C), stained in Gomori's trichrome, mounted in Canada balsam	+
Diplectanum bauchotae	*Sphyraena flavicauda*	Specimen fixed in AFA, mounted in glycerin gelatin	−
Pseudohaliotrema sp.	*Siganus rivulatus*	Specimen fixed in hot 5% formalin (60 C), stained in Gomori's trichrome, mounted in Canada balsam	+
Pseudorhabdosynochus sp.	*Epinephelus marginatus*	Specimen fixed in hot 5% formalin (60 C), stained in Gomori's trichrome, mounted in Canada balsam	+
Kuhnia scombri	*Scomber scombrus*	Specimen fixed in hot 5% formalin (60 C), stained in Gomori's trichrome, mounted in Canada balsam	+
Cemocotylella sp.	*Caranx melampygus*	Specimen fixed in hot 5% formalin (60° C), stained in Gomori's trichrome, mounted in Canada balsam	+
Cemocotylella sp.	*Caranx melampygus*	Specimen fixed in AFA, stained in Gomori's trichrome, mounted in Canada balsam	+

without destruction of the specimen. LSCFM, however, is not without disadvantage and may introduce some artifacts. For example, Klaus et al. (2003), who examined insect genitalia, indicated that axial distortion, particularly along the z-axis, may be associated with LSCFM when observing thick specimens. However, comparison of LSCFM images of monogenoidean sclerites with respective sclerites of other specimens oriented in the same plane as those of the LSCFM images suggests that axial distortion is minimal, probably owing to their comparatively smaller size and thickness.

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Failure to Propagate *Cryptosporidium* spp. in Cell-Free Culture

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ABSTRACT: The successful propagation of *Cryptosporidium parvum* in cell-free culture medium was recently reported. To investigate whether this phenomenon could be broadened to include other *C. parvum* isolates, as well as *Cryptosporidium hominis*, we attempted to propagate 3 isolates in cell-free medium under reported culture conditions. *Cryptosporidium* oocysts from *C. parvum* strains Moredun (MD) or IOWA or *C. hominis* strain TU502 were added to media containing coagulated newborn calf serum. The cultures were sampled at various times throughout a 45 (IOWA) or 78 (MD, TU502)-day period and were microscopically examined for various life stages of *Cryptosporidium*. Cell-free cultures harvested on days 45 and 68 postinoculation were tested for in vitro infectivity on Madrin-Darby bovine kidney cells. In vivo infectivity testing was performed using either infant or 2-wk-old immunosuppressed C57BL mice with cell-free cultures harvested on days 52 and 78. Fecal and gut samples collected from mice were examined by modified acid-fast staining. Data from wet mounts, electron microscopy, and in vitro and in vivo infectivity testing showed that the original oocysts did not complete their life cycle and produce new, viable, infectious oocysts in cell-free culture. Thus, we conclude that this is not a universal phenomenon or readily accomplished.

Early last year, a landmark publication appeared in which the successful completion of the life cycle of *Cryptosporidium parvum* in host cell-free culture was reported (Hijjawi et al., 2004). The previous milestone achievement in this area was in 1984, with the successful growth of *C. parvum* in cell culture (Current and Haynes, 1984). However, cell culture growth is largely limited to the asexual phase and, consequently, does not lead to immortalization. Coupled with the inability to cryopreserve the parasite, these 2 key obstacles have hindered progress in laboratory investigations on this parasite comparable to those achieved with other Apicomplexa. Therefore, the accomplishment reported by Hijjawi et al. (2004) was received with much enthusiasm and excitement by the scientific community, if somewhat with astonishment. Astonishment, because intracellular eukaryotic protozoans, Apicomplexa in particular, depend on the host-cell machinery without which they cannot complete their life cycle.

We have set out to repeat the experiments reported by Hijjawi et al. (2004) with the hope of finally immortalizing this parasite once and for all, a task many scientists have been trying unsuccessfully to accomplish over the past 2 decades. We report here, however, our failure to propagate 2 isolates of *C. parvum* and 1 of *Cryptosporidium hominis* in cell-free medium, having followed the exact procedures described by Hijjawi et al. (2004).

Freshly derived oocysts of the zoonotic *C. parvum* isolates Moredun (MD; Okhuysen et al., 2002) and IOWA and the human *C. hominis* isolate TU502 (Akiyoshi et al., 2002) were purified and stored in phosphate buffered saline (PBS) containing penicillin G (5,000 U/ml) and streptomycin (5 mg/ml) at 5 C. The IOWA isolate was originally derived from a calf and was collected by Dr. Harley Moon (Iowa State University, Ames, Iowa). It has been repeatedly passaged in calves in the laboratory of Dr. Charles Sterling (University of Arizona, Tucson, Arizona). Before culturing, oocysts were excysted, using freshly prepared, filtered hydrochloric acid water (pH 2.5) containing trypsin (0.5% v/v). Oocysts (2×10^7) were added to the excystation solution and incubated at 37 C for 20 min, with mixing every 5 min. The oocysts were centrifuged at 5,000 *g* for 5 min and resuspended in 10 ml of maintenance media (500 ml of RPMI-1640 containing 1% fetal calf serum, 0.15 g l-glutamine, 1.5 g sodium bicarbonate, 0.1 g bovine bile, 0.5 g glucose, 125 μg folic acid, 500 μg 4-aminobenzoic acid, 250 μg calcium pantothenate, 4.38 mg ascorbic acid, 5 ml of pen/strep and 1.8 g HEPES, adjusted to pH 7.4).

To promote *Cryptosporidium* spp. development in vitro, filtered maintenance media, with a semisolid phase consisting of coagulated newborn calf serum, was used. Newborn calf serum (10 ml) was added to each 25 cm^2 flask and incubated for 45 min at 70–80 C in a water bath. The serum was allowed to cool and solidify before maintenance media, and excysted oocysts were added. In total, 23 flasks were used for this study to investigate the propagation of 3 different *Cryptosporidium* spp. isolates. Twenty-one flasks contained the semisolid phase and 40 ml of maintenance media. For controls, 1 flask contained 40 ml of maintenance media without coagulated serum and oocysts, and another flask contained 40 ml of maintenance media plus 3×10^6 oocysts. Eight flasks were inoculated with either 10^6 (4 flasks), 2×10^6 (2 flasks), or with 3×10^6 oocysts (2 flasks) of *C. hominis* TU502 isolate. A similar set of flasks was inoculated with oocysts of the *C. parvum* MD isolate. The last 5 flasks were inoculated with 3×10^6 oocysts of the *C. parvum* IOWA isolate. All flasks were incubated at 37 C in a 5% CO_2 incubator. No bacterial or fungal contamination was visually observed in any of the 23 flasks during the study period.

The contents of the flasks were sampled on a rotating basis, except for 2 flasks containing the highest inocula for each isolate, which were left undisturbed until the end of the experiment. Flasks were sampled 2–3 times per wk over a period of 78 days (45 days for the IOWA isolate) by removing 10 ml of media from each flask and centrifuging at 4,000 *g* for 15 min. The resulting pellet was resuspended in 1 ml of media, and wet mounts were prepared and examined microscopically. Wet mount preparations from flasks sampled during the first 2 wk showed intact oocysts, empty shells, and motile sporozoites. As the experiment progressed, there was an increase in the clumping and number of shells. Some motile sporozoites were also noted. When the experiment was terminated on day 78 (day 45 for IOWA), a high concentration of empty shells and some intact oocysts were observed, but no motile sporozoites were seen (Fig. 1A).

The contents of the flasks were also examined for the presence of oocysts by indirect immunofluorescence (Merifluor *Cryptosporidium*/*Giardia* IFA Kit, Meridian Biosciences, Cincinnati, Ohio) and by elec-

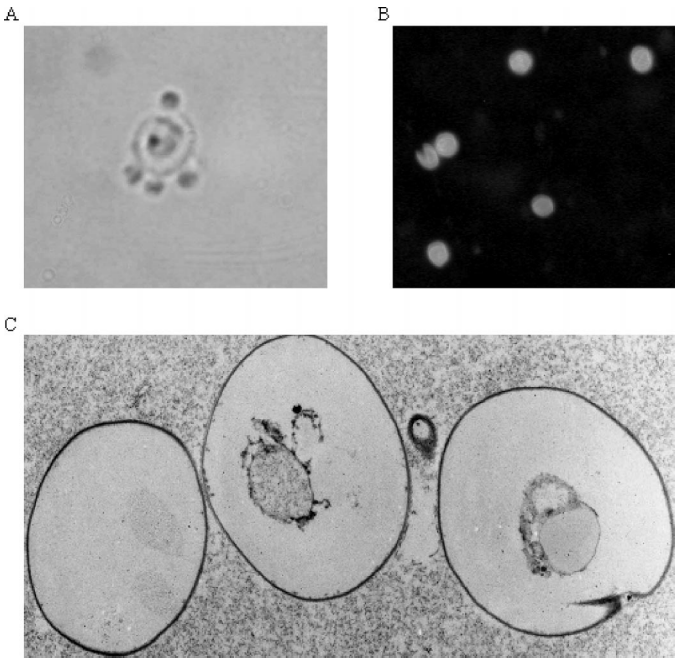

FIGURE 1. Microscopic analysis of *Cryptosporidium* spp. in cell-free culture. **(A)** Wet mount of 13-day-postinoculation *C. hominis* oocyst with 3 sporozoites still attached. **(B)** Immunofluorescence of day 64 IOWA oocysts showing old, intact, and broken-open oocysts. **(C)** Scanning electron micrograph of day 65 IOWA oocysts showing oocyst shell (left) and oocyst shells with centrum (center, right).

tron microscopy. Smears were made for IFA and examined microscopically on day 13 (TU502, MD), day 43 (TU502, MD), and day 51 (IOWA). Also, on day 64 (IOWA) and day 78 (MD, TU502, controls), all remaining flasks (except for 2 flasks for each isolate, which were not sampled) had their contents centrifuged and the corresponding pellets examined by IFA. IFA slides showed fragments of oocysts, empty shells, and some intact oocysts for all days examined (Fig. 1B). However, the intact oocysts were unlike those seen in the control (oocysts not maintained in cell-free culture) slides. Experimental oocysts were smooth in appearance in contrast to the ''raisinlike'' appearance of the control oocysts. It is suspected that the experimental oocysts seen were actually oocysts from the original inoculum. On day 47 (MD and TU502) and day 65 (IOWA), the contents of 1 set of flasks, originally inoculated with the highest number of parasites and not sampled during the experiment, were collected by centrifugation (4,000 *g*, 15 min), fixed, embedded, sectioned, and examined by electron microscopy. Carefully examined electron micrographs of ultrathin sections also revealed various stages of decaying oocysts; a few were morphologically intact. Most, however, were open with centrum either still within or forming part of the debris (Fig. 1C).

In addition, the contents of the cultures from day 43 (MD and TU502) and day 51 (IOWA) were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the *Cryptosporidium* spp. oocyst wall protein (COWP) to check for the presence of parasite DNA. DNA was extracted from the pellets using the FastDNA SPIN Kit for Soil (Qbiogene, Inc., Carlsbad, California). COWP PCR-RFLP analysis was performed as described by Akiyoshi et al. (2002). All 3 cultures showed the presence of *Cryptosporidium* DNA, which is most likely from dead sporozoites or oocysts.

Finally, the cell-free cultures of the 3 isolates were tested for infectivity in vitro and in vivo. For the in vitro assay, infectivity was tested on Madrin-Darby bovine kidney (MDBK) monolayers grown in 96-well plates as previously described (Tzipori et al., 1994; Tzipori, 1998). The cell-free cultures were harvested on day 47 (IOWA) and day 68 (MD and TU502) by centrifugation (4,000 *g*, 15 min) and the pellets resuspended in fresh media, which were then used to infect the MDBK cells. After 48 hr at 37 C (5% CO_2), the cells were fixed, stained with FITC, and examined by immunofluorescence microscopy. None of the cell-free cultures contained any identifiable endogenous parasite forms, but oocysts in various stages of decay were observed. As a positive control, some of the excysted oocysts originally used to inoculate the cell-free cultures were also tested for infectivity on MDBK cells. These oocysts were found to infect MDBK cells, thus, demonstrating the viability and infectivity of the original oocyst preparation.

The cell-free cultures were assayed in vivo for the presence of infectious oocysts using 2 mouse models (Tzipori, 1998). Two-week-old C57BL mice were subcutaneously injected with dexamethasone (0.33 mg, 20 μl) once every other day for a course of 3 injections. After the last injection, mice were orally inoculated with the contents of the second set of flasks containing the highest inoculum for IOWA (day 52, 5 mice), MD (day 78, 5 mice), or TU502 (day 78, 5 mice), which were harvested by centrifugation. The contents of previously sampled flasks (day 78, MD and TU502) were also used to inoculate 4 infant mice per isolate (Tzipori, 1998). Fecal samples were examined by modified acid-fast staining 4 days postinoculation (PI) and subsequently every other day for 14 days. After 14 days PI, mice were killed by CO_2 asphyxiation, and gut contents were harvested, homogenized, and resuspended in PBS. The gut samples were again examined for oocysts by modified acid-fast staining. Neither the fecal smears nor gut samples from either the immunosuppressed or infant mice showed the presence of oocysts during this 2-wk-period, thus, indicating that none of the mice experienced a *Cryptosporidium* spp. infection. This was further confirmed by PCR-RFLP analysis of the guts of the 2-wk-old and newborn mice, which were negative for the presence of parasite DNA.

We attempted to propagate 3 different strains of *Cryptosporidium* in cell-free culture. Cultures were monitored up to 45 (IOWA) and 78 (MD and TU502) days PI by wet mount and IFA slide analyses, PCR-RFLP analysis, electron microscopy, and in vitro and in vivo analysis. None of the slides, micrographs, or assays showed any indication of the original oocysts completing their life cycle and producing new, viable, infectious oocysts. Thus, having followed to the letter, the exact method described by the investigators (Hijjawi et al., 2004) from Murdoch, Australia, we were disappointed with our inability to repeat their claims with either of the 2 species of *Cryptosporidium* used. However, this is not all that surprising because apicomplexan parasites are not known to propagate outside the host cell. We are not sure how to interpret the observations made by Hijjawi et al. (2004) because they did not provide genetic, antigen-specific, or ultrastructural evidence of parasite presence. We conclude, therefore, that if the observations made by Hijjawi et al. (2004) are at all correct, cell-free cultivation of *Cryptosporidium* spp. is not a universal phenomenon or readily accomplished.

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