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Giemsa Staining and Antibody Characterization of *Colpodella* sp. (Apicomplexa)

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Abstract

Colpodella species are free-living alveolates that possess an apical complex used for attaching to eukaryotic prey protists for ingestion of the cytoplasmic contents of the prey. *Colpodella* sp. are the closest relatives of the Apicomplexa, a phylum that includes the important human pathogens *Plasmodium falciparum, Toxoplasma gondii* and *Cryptosporidium parvum*. In this study, we investigated morphological characteristics of *Colpodella* (ATCC 50594) in a diprotist culture containing *Bodo caudatus* as prey in order to identify features differentiating both protists. The level of apical complex protein conservation among free living alveolate relatives of apicomplexans and intracellular apicomplexan pathogens is unknown. Antibodies against proteins of the apical complex in *Colpodella* sp. are currently unavailable. We performed staining and immunological characterization of *Colpodella* in a diprotist culture containing *B. caudatus* to aid routine differentiation of predator and prey in culture. Staining revealed distinguishing morphological features of both protists. The kinetoplast in *B.caudatus* was identified using Giemsa staining and was used to differentiate *B. caudatus* from *Colpodella sp*. trophozoites. We show for the first time predator-prey interactions identified by Giemsa staining. We also show that antibody specific for *P. falciparum* RhopH3, a high molecular weight rhoptry protein, reacts specifically with a protein in the apical complex of *Colpodella.* This reactivity demonstrates conservation of rhoptry proteins between *Plasmodium* and *Colpodella sp*.

Keywords: Apical complex organelles; *Colpodella*; *Plasmodium falciparum*; Rhoptries; Rhoptry proteins; RhopH3.

Introduction

The free-living alveolate, *Colpodella* is the closest relative of the Apicomplexans [1]. Organelles of the apical complex for which the phylum is named, aids parasite entry into red blood cells, lymphocytes and epithelial cells in human and animal hosts. The organelles are the subject of intense research for diagnostics, vaccine and drug discovery in malaria. Apical complex organelles include the rhoptries, micronemes, dense granules, polar rings and conoid which are used for host cell attachment and invasion by apicomplexans. The conoid is open in *Colpodella* and is referred to as a pseudoconoid. Among the apicomplexans, some invasive stages lack a conoid and in some dinoflagellates the conoid is absent [1]. *Colpodella* and *Plasmodium* share the presence of apical complex organelles with free living photosynthetic and non-photosynthetic predatory organisms such as *Colponema*, *Chromera, Vitrella* and *Aerocoleus* species [1-7] and intracellular dinoflagellate organisms of *Perkinsus* species [8]. Recent investigations of coral reef environments have revealed organisms closely related to apicomplexans with a lineage designated Apicomplexan Related Lineage-5 (ARL-V) now shown to be phylogenetically the closest to the apicomplexans [6,9]. The apical complex in *Colpodella* is similar to that of *Plasmodium* [10,11]. Rhoptries in *Colpodella* sp. are varied morphologically [12]. In some species rhoptries are described as elongated with "bulbous" ends and narrow necks, similar to merozoite rhoptries in apicomplexans. Additionally, "lentil-shaped" rhoptries were also described for *C. vorax* [12]. Rhoptry contents are discharged following contact with host cells among the apicomplexa [5]; a process also observed in *Colpodella* sp. The apical end, specifically the rostrum containing the pseudoconoid is used by *Colpodella* to attach to eukaryotic prey protists enabling *Colpodella* to aspirate the cytoplasmic contents of the prey in a process known as myzocytosis. *Colpodella gonderi* and *C. tetrahymenae* are ectoparasites of ciliates where the rostrum is used to attach to the surface of the ciliates. The ciliates *Colpoda steinii*, *Pseudoplatyophrya nana* and *Grossglockneria acuta* are parasitized by *C. gonderi* [13]. *Colpodella tetrahymenae* feeds on the ciliate *Tetrahymenae aff. pyriformis* [14]. There is limited genomic and proteomic data for the free living alveolates such as *Colpodella*. A recent study examined the mRNA (transcriptome) sequence of two chromerids and three colpodellids [15], and found many "apicomplexan-specific" orthologous genes, present in the free-living relatives; including genes encoding rhoptry, microneme, parasitophorous vacuole and merozoite surface proteins [15]. Conserved extracellular protein domains have also been described for free-living alveolates and apicomplexans

[16]. The subcellular organization and distribution of proteins conserved among apicomplexans, colpodellids and dinoflagellates such as *Perkinsus* have not been identified. To the best of our knowledge, there are no antibodies against proteins of the apical complex among the colpodellids and the distribution of proteins associated with predation, host attachment and myzocytosis is unknown. Furthermore, detailed characterization of the *Colpodella* sp. and the prey protist *B. caudatus* by staining as a means of distinguishing both protists in culture is not routinely performed. In order to explore the feasibility of employing *Colpodella* as a heterologous model to investigate rhoptry biogenesis, organization and the function of genes encoding apical complex proteins, we evaluated diprotist culture conditions and staining characteristics of the protists *Colpodella* and *B. caudatus* using different fixation conditions for Giemsa staining. Due to the similarities in the attachment, "junction" formation and discharge of apical organelle contents following interaction of the rostrum with the plasma membrane of *B. caudatus*, we hypothesized that antibodies specific to *Plasmodium* rhoptry proteins would cross react with *Colpodella* proteins. We therefore performed cellular localization and antibody characterization of *Colpodella* trophozoites using an antibody specific for the *P. falciparum* 110 kDa high molecular weight rhoptry protein, RhopH3 (gene ID PF3D7_0905400) (Plasmodb.org) by immunofluorescence assay (IFA). We show for the first time, predator-prey interactions between *Colpodella* sp. and *B. caudatus* and rhoptry protein conservation between *P. falciparum* and an ancestral free-living alveolate. We identified distinct vesicles within the apical complex of *Colpodella* sp. reactive with anti-RhopH3 antibody and also identified the cellular distribution of RhopH3 in *Colpodella* trophozoites.

Materials and Methods

Diprotist Culture Conditions

Colpodella sp. (ATCC 50594) (Manassas, Virginia, USA) was cultured with prey species *B. caudatus* in Hay medium (Wards Scientific) (Rochester, New York, USA), bacterized with single colonies of *Enterobacter aerogenes* in 25 cm^2 or 75 cm^2 tissue culture flasks (Corning) containing 10 ml and 30 ml cultures, respectively. *Enterobacter aerogenes* was cultured on nutrient agar plates. Bacterized medium was inoculated with diprotist culture scraped with a 25 cm cell scrapper (Falcon), with a 1.8 cm blade. Ten ml cultures were inoculated with 0.5 ml resting cysts and 30 ml cultures with 1.5 ml resting cysts. Cultures were incubated at 22-24 °C. Cells in tissue culture flasks were observed using an inverted microscope (Nikon TMS, Type 104) under phase microscopy to determine the level of predator-prey interactions and the increase in cell density. An "attack index" was determined where the number of prey attacked by one or more predators was counted as a means of ascertaining the activity of the trophozoites in culture and also determining the number of *Colpodella* sp. in culture (Table 1). The predator to prey ratio in ten fields was counted in a wet mount prepared from cells obtained from pellets after centrifugation of 2 ml of diprotist culture. Counts were performed in triplicate from 10µl suspensions obtained after centrifugation of cultures pooled and resuspended in 100 µl Hay medium. Detain (Ward's) was added to wet mounts to slow down protist motility. Counts of unattached cells were also performed. All cultures were maintained aseptically. No antibiotics or filtration was used to eliminate bacteria in the medium.

# of Frames / *Colpodella* Attacking *Bodo*	1	2	3	4	5	6	7	8	9	10
1 *Colpodella* attacking 1 *Bodo caudatus*	23	19	20	23	22	22	21	20	22	23
2,3 & 4 *Colpodella* attacking 1 *Bodo caudatus*	13	10	11	9	8	8	10	8	8	13

Table1: Attack index of predator prey interactions observed in wet mounts of diprotist cultures containing *Colpodella* sp. and *B. caudatus*

Giemsa Staining and Light Microscopy

Diprotist cultures were harvested and placed directly into eppendorf tubes and centrifuged at 13,000 xg in a microfuge (Biofuge 13, Heraeus Instruments) for 10-15 min or cells were placed in 15 or 50 ml sterile tubes, centrifuged at 1475xg for 10 min in an IEC HN-SII table top centrifuge, the supernatants were discarded and the pellets resuspended, transferred to eppendorf tubes and centrifuged at 13000 xg in a microfuge. Supernatants were examined for the presence of organisms before discarding. For light microscopy, cells were fixed in absolute methanol alone for 1 min, fixed in 0.5%, 2% or 5% formalin in Eppendorf tubes for 10 min on ice or in 5% formalin directly in T-flasks for 10 min at room temperature. Diprotist culture samples from resuspended pellets were also smeared on glass slides, air-dried and heat fixed by passing the slide over a Bunsen burner flame. Fixed cells in culture flasks were centrifuged as indicated above. Following the removal of supernatant, the pellet was washed once in 1x PBS and resuspended in 0.1 ml of 1x PBS. Formalin fixed smears were prepared on glass slides and air-dried before staining with Giemsa stain (0.4% stock in buffered methanol, pH 6.8) (Sigma-Aldrich, St. Louis, MO). Smears for methanol fixation were prepared directly on glass slides and air-dried before fixation. Giemsa staining was carried out for 1, 2, 5, 10, 15 or 25 minutes using different concentrations (0.1%, 0.02%, 0.04% and 0.08%) of Giemsa. In order to observe and record cells, wet mounts were also prepared with the addition of 0.5 % formalin alone or Detain (Wards) to slow down the movement of both protists. Cells fixed with 0.5% formalin were still actively motile. Cell swimming, feeding, cyst formation and excystation were observed in culture using an inverted microscope and in wet mounts on glass slides. The diprotist culture containing bacteria was processed for smears and staining. Images of wet mount and Giemsa-stained cells were captured using an Olympus CX31 microscope with an Olympus SPOT IDEA U-TVO.5XC-3 camera attachment and analysis performed using SPOT imaging BASIC version 5.3, 2014 Software. Giemsa stain and wet mount images were also adjusted to 300 dpi using the CYMK color mode on Adobe photo shop (CS6).

Immunofluorescence and Confocal Microscopy

Immunofluorescence and confocal microscopy was performed on *P. falciparum* infected erythrocytes and a *Colpodella* sp. and *B. caudatus* diprotist culture as described previously [17]. Cells were fixed using absolute methanol or acetone. Briefly, smears were fixed in cold methanol alone or methanol and acetone combinations for 5 min at -20 °C followed by incubation with rabbit and mouse polyclonal antibodies or mouse monoclonal antibodies; either individually or together in colocalization experiments. For colocalization assays, mouse primary antibodies (*Physarium polycephalum* anti-β-tubulin monoclonal antibody KMX-1 [18], 1:1000 dilution, anti-His-FLPbRhop-3 [19], 1:200 dilution and rabbit primary antibodies anti-*P. falciparum* recombinant RhopH3 (gene ID PF3D7_0905400) (Plasmodb.org) antiserum 686 [20] and anti-*P. falciparum* whole rhoptries, antiserum 676 [21] diluted 1:100 were used as primary antibodies. The secondary antibodies directed to both species, conjugated to different colored fluorochromes, were used for detection of primary antibodies. Incubation with primary antibodies was carried out for 1 h; slides were washed three times with 1×PBS followed by incubation for 1 h with secondary mouse or rabbit antibodies conjugated to Alexa 488 or Alexa 633 diluted 1:1000 (Molecular Probes, ThermoFischer Scientific). The smears were washed three times with 1×PBS followed by one wash in distilled water supplemented with 8μg/ml bis-benzimide and before the Incubations were performed at 37 °C. Vectashield containing 4', 6-diamidino-2-phenylindole (DAPI; Vector, Burlingame, CA, USA) was used to mount the slides. Images were collected using a Leica TCS-SP5II upright laser scanning confocal microscope (Leica Microsystems, GmbH, Wetzlar, Germany). Normal (preimmune) mouse or rabbit serum (NMS and NRS, respectively), were used as negative controls for IFA. Giemsa stained and confocal images were adjusted to 300 dpi using the CYMK color mode and RGB color mode on Adobe photo shop (CS6). ImageJ was used to change the channel from magenta to red in the IFA images. No brightness or contrast adjustments were made. Resolution (1024×1024) was divided by the physical distance (32.05 micrometers) to calculate pixel per micrometer. The result was 26 pixels per micrometer. The image size was 5 micrometers so the scale is 130 pixels per micrometer. Confocal microscopy was performed at the Cleveland Clinic, Lerner Research Institute Imaging Core, Cleveland, OH, USA.

Results

Wet Mounts of Diprotist Culture

An "attack index" was generated to facilitate counting of protists in culture to obtain a ratio of predator and prey in culture (Table 1). The average number of predator attacks on prey counted in ten fields, in triplicate from a 10μl volume from a pellet resuspended in 100 μl is shown in Table 1. Single predator-prey (1:1) interactions were the most common. However, multiple (2:1, 3:1 and 4:1) attacks of predator on prey were also observed with up to four *Colpodella* observed attached to a single *B. caudatus* prey. With the addition of Detain (Ward's) to slow down cells, unattached cells could also be counted in each field. The presence of prey surrounded by two or more predators was indicative of very active cultures.

In order to distinguish each protist in culture, wet mounts and Giemsa stained preparations of fixed cells were prepared. In wet mounts of the diprotist culture, individual *Colpodella* and *B. caudatus* were observed (Figure 1A). Predator-prey interactions were also observed in culture (Figure 1, panels B and C). Black arrowheads identify *Colpodella* trophozoites attached to *B. caudatus* (panels B and C). Smears from the pellets of diprotist culture prepared following centrifugation were fixed using heat, absolute methanol or formalin (0.5%, 2% or 5%). Cells fixed with 0.5% formalin were still very active with rapid motility observed for both protists. Smears prepared directly from culture or from re-suspended pellets, air dried and stained with Giemsa failed to consistently show flagella due to "balling up" response of the cells where it appeared the cells rounded up following application of cells on glass slides and air drying, making it difficult to visualize the flagella.

We wanted to routinely distinguish *Colpodella* sp. from *B. caudatus* in culture and determine the ratio of predator to prey in culture. When cells were fixed directly with 5% formalin, centrifuged and the pellets re-suspended in 1x PBS, cells were stained with Giemsa and the flagella along with individual cells and predator-prey interactions could be observed. Figure 2, panel A, shows predator-prey interactions identified following formalin fixation and Giemsa staining. The green arrowheads are shown near *Colpodella* trophozoites attached to *B. caudatus* (black arrowheads). In the two top interacting pairs, vacuoles were seen in the cytoplasm of *Colpodella*, (Figure 2, top left, panel A). Flagella from the predator and prey were observed extended from the anterior ends of each cell (Figure 2, panel A). In initial predator attacks, Colpodella appeared vacuolated with a lighter staining cytoplasm. With prolonged contact, cells became fusiform and more darkly stained. Protrusion of the conoid was observed and an attachment "junction" was observed in many interactions between the predator and prey (Figure 2A) with an increase in size as the prey was attacked and the cytoplasm aspirated. Red arrowheads mark two of the junctions formed between predator and prey showing the tight connection between the rostrum of *Colpodella* and plasma membrane of *B. caudatus*. Individual *Colpodella* sp. and B. caudatus cells are shown in Figure 2, panels B and C, respectively. The black arrowhead shows the nucleus (n) for Colpodella and *B. caudatus* and kinetoplast (k) of *B. caudatus*. In cells stained with Giemsa, the nucleus could be seen within the cytoplasm along with cytoplasmic vacuoles and flagella (yellow arrowhead) in both *Colpodella* and *B. caudatus* cells. Giemsa staining of 1 to 2 minutes showed better discrimination of cytoplasmic structures within cells. Stained bacterial cells were also observed in the background.

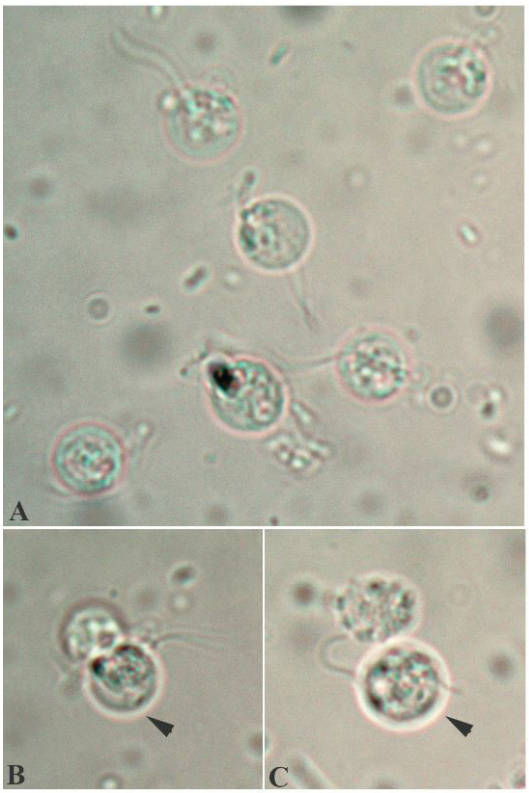

Figure 1: Wet mount of a diprotist culture maintained in Hay medium containing *Colpodella* (ATCC 50594) and *B. caudatus*. Cells were centrifuged and the pellet resuspended in Hay medium for wet mount preparation. Detain (Ward's) was added to wet mounts to slow protist motility. A. wet mount of diprotist culture showing *Colpodella* trophozoites and *B. caudatus*; B and C. Wet mount of *Colpodella* (black arrowhead) attached to *B.caudatus*. (1000x magnification)

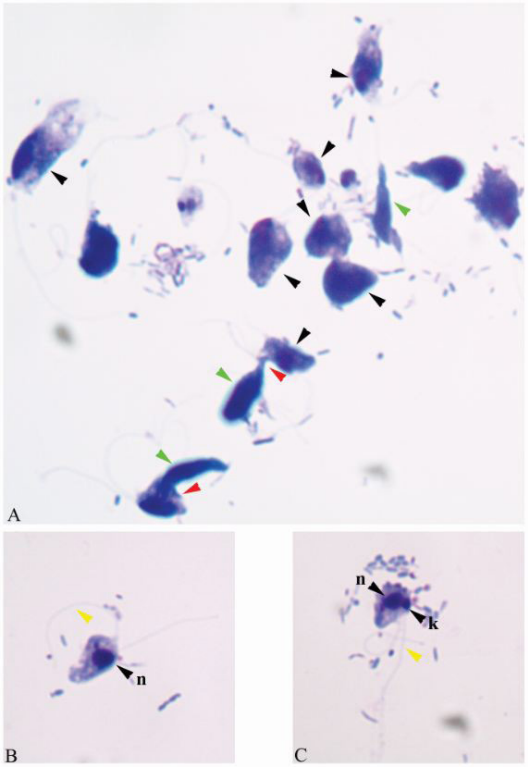

Figure 2: Giemsa stain of *Colpodella* sp. and *B. caudatus*. The diprotist culture was centrifuged and the pellet fixed in 5% formalin, centrifuged, and the pellet washed in 1x PBS. The pellet was resuspended in 1x PBS, smears were prepared on glass slides, air-dried and stained with Giemsa stain. A. Giemsa stain of the diprotist culture showing five predator- prey attacks with the green arrowheads identifying the predator *Colpodella* attached to the *B. caudatus* prey (black arrowhead). Predator-prey pairs shown in the top of the panel, show early stages of attack with vacuoles present in the cytoplasm of *Colpodella*. In the three other predator-prey pairs, *Colpodella* is darkly stained, elongated and fusiform with tight connections or "junctions" formed with the anterior apical end of the trophozoites (red arrowheads). Unattacked *B. caudatus* cells and *Colpodella* trophozoite were also observed. Clusters of bacterial cells were also observed in the smear. B. Individual Giemsa stained *Colpodella* trophozoite, black arrowheads identify flagella (yellow arrowhead); C. Individual Giemsa stained *B. caudatus*, show flagella (yellow arrowhead), the nucleus (n) and kinetoplast (k). (1000x magnification)

Immunofluorescence and confocal microscopy

Methanol fixed smears from diprotist cultures were reacted with antiserum 686 [20], specific for the 110 kDa high molecular weight rhoptry protein RhopH3 (gene ID PF3D7_0905400) (Plasmodb.org). Antiserum 686 was incubated together with antibodies against β-tubulin [18]. We wanted to know if antibodies specific for a *P. falciparum* rhoptry protein would cross react with proteins in *Colpodella* sp. Antiserum 686 (green) reacted with "doughnut" shaped vesicles distributed within the cytoplasm of *Colpodella* (Figure 3, panels A-D). Antiserum 686 also reacted diffusely with the cytoplasm in addition to the vesicles. Anti-β-tublin antibody (red) reacted with the cell body and flagella of *Colpodella* but only with the cell body of *B. caudatus* (Figure 3, panels E-H). The DAPI stained nucleus and kinetoplast of *B. caudatus* were observed (Figure 3 panels E and F). However, there was no reactivity of the RhopH3 specific antibody with *B. caudatus* proteins (Figure 3, panel H) demonstrating that the cross-reactivity of the *Plasmodium* specific antibody with *Colpodella* proteins is highly specific to *Colpodella* sp. proteins. Antibodies against whole rhoptries of *P. falciparum* and *P. berghei* [19,21] also reacted with the same vesicular structures (results not shown). Figure 3, panels I-L are negative controls with normal rabbit and mouse serum showing that there was no protein reactivity by the negative control serum on *Colpodella* sp. or *B. caudatus*. Anti *P. falciparum* RhopH3 reactivity is shown with *P. falciparum* segmented schizonts showing the punctate staining of merozoite rhoptries in a positive control of the rhoptry specific antibodies evaluated (Figure 3, panels M-P; Figure S1, panels A-D). Faint anti-β-tubulin antibody reactivity was observed on *P. falciparum* merozoites (Figure 3, panel O). Figure 3 panels M-P show fully segmented *P. falciparum* schizonts with the punctate staining characteristic of blood stage merozoites. In mature schizonts not fully segmented, antiserum 686 reactivity was also observed in the developed merozoites within the schizont (Supplementary Figure 1).

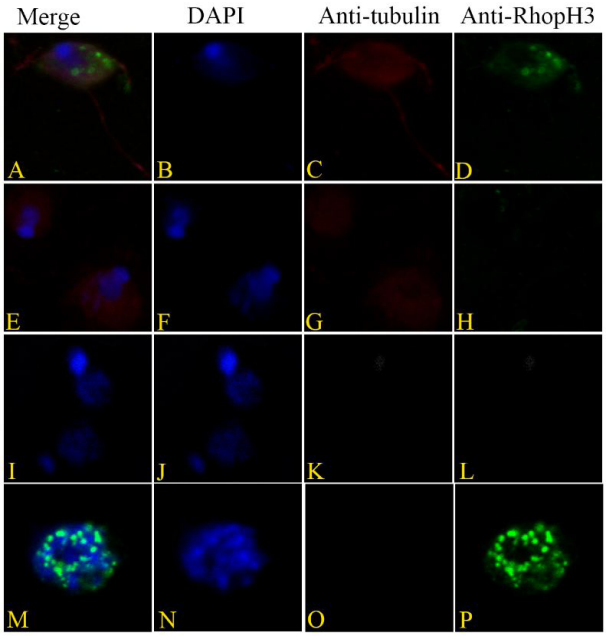

Figure 3: Indirect immunofluorescence assay (IFA) of a diprotist culture containing *Colpodella* sp and *B. caudatus* and IFA of *Plasmodium falciparum* schizonts. Antiserum 686 specific for the high molecular weight *P. falciparum* rhoptry protein RhopH3 and anti-β-tubulin monoclonal antibody KMX-1 of Physarum polycephalum. Samples of the diprotist culture containing *Colpodella* (ATCC 50594) and *B. caudatus* were fixed with absolute methanol. Panels A-D. Diprotist culture was incubated with antiserum 686 (1:100; green) and anti-β-tubulin KMX-1 (1:1000; red) followed by incubation with ALEXA-488 Goat anti-rabbit antibody (1:1000) and ALEXA 633 Goat anti-mouse antibody (1:1000). Smears were mounted with Vectashield containing DAPI for nuclear staining. Panel A. Merge of antiserum 686, KMX-1 and DAPI showing 686 antibody reactivity with cytoplasmic vesicles in the anterior section of *Colpodella* trophozoites and the apical tip of the cell; B, DAPI nuclear staining, C, KMX-1 reactivity on cell body and flagella of *Colpodella* trophozoite (red) and D, antiserum 686 reactivity (green) showing protein recognition and reactivity of the cytoplasm and vesicles. Panels E-H. No RhopH3 antibody reactivity was observed with *B. caudatus* cells. Merge of antiserum 686, KMX-1 and DAPI (E) showing DAPI and anti-β-tubulin staining with no 686 antibody reactivity. Panel F shows DAPI staining of nucleus and kinetoplast of B. caudatus. Panel C shows reactivity of anti-β-tubulin antibody KMX-1 with the cell body of *B. caudatus* with no flagella staining. Panel H shows no reactivity of antiserum 686 with B. caudatus. Panels I-L. Diprotist culture was incubated with normal rabbit (1:100) and normal mouse serum (1:200) followed by incubation with ALEXA-488 Goat anti-rabbit antibody (1:1000) and ALEXA 633 Goat anti-mouse antibody (1:1000). Smears were mounted with Vectashield containing DAPI for nuclear staining. I, merge showing DAPI staining and no protein reactivity, J, DAPI alone, K and L. no protein reactivity on *Colpodella* trophozoites or *B. caudatus* with normal rabbit and mouse serum. Panels M-P. Segmented *P. falciparum* (strain 3D7) schizonts fixed in ice-cold methanol and acetone (1:1 ratio) were incubated with antiserum 686 (1:100; green) and KMX-1 (red). Merge of DAPI, RhopH3 and β-tubulin showing antibody reactivity with merozoite rhoptries showing punctate staining of rhoptries (M), DAPI nuclear staining (N), β-tubulin (O) and antiserum 686 reactivity alone (P). (3,780 x magnification)

Discussion

In this study, we evaluated fixation and staining protocols to visualize *Colpodella* sp. (ATCC 50594) and *B. caudatus* in culture. We also used an antibody specific for RhopH3, a *P. falciparum* merozoite rhoptry protein, to identify protein distribution in the apical complex region of *Colpodella* sp. Due to the feeding behavior described for *Colpodella* regarding the attachment of the rostrum to prey during myzocytosis [10,12], we hypothesized that rhoptry proteins would be conserved between *Colpodella* sp. and *P. falciparum*. Light, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) studies show detailed

features of the cellular organization of *Colpodella* species, and the presence in the apical complex of a pseudoconoid, rhoptries and micronemes [10-12, 22]. Trichocysts displaying diverse morphological forms are also present in the anterior ends of some *Colpodella* sp. [11,14]. Several species of *Colpodella* have been described [10], with some species reassignments carried out due to new morphological, molecular and phylogenetic data [14]. Differences in morphology with respect to the presence of a ventral gutter in the anterior end of the cells in some species were also noted [10]. For routine culture and investigation of *Colpodella*, a staining protocol is needed in addition to wet mount images to allow for consistent differentiation of predator and prey. These types of protocols are currently unavailable. In this study, cells were processed for Giemsa staining following fixation with heat, methanol and formalin. Each fixation method preserved cell morphology and stained cells could be examined. However, methanol and formalin fixation allowed for clearer differentiation of cytoplasmic contents. Formalin fixation allowed for detection of flagella following Giemsa staining. The points of attachment during feeding by *Colpodella* sp. were observed by Giemsa staining (Figure 2A), confirming the observations seen in wet mounts (Figure 1). Additionally, the morphological differences in both protists could also be identified. Cells were also fixed in acetone for IFA but displayed poor morphology when examined (results not shown).

Differentiation of protists in culture will facilitate cell biological, molecular and biochemical analysis. We demonstrated in earlier studies, that the 110 kDa RhopH3 (also Rhop3) high molecular weight rhoptry protein is secreted into the erythrocyte host membrane during merozoite invasion [23]. RhopH3 forms a complex with RhopH2 and RhopH1/Clag proteins following their synthesis in the blood stage of *Plasmodium*. RhopH3 has a dual role in merozoite invasion and nutrient uptake in *P. falciparum* [24]. RhopH3 also participates in the formation of the plasmodial surface anion channel (PSAC) [24]. The RhopH3 gene is essential to *P. falciparum* development in the blood stage as it is refractory to deletion [25]. The attachment of *Colpodella* to *B. caudatus* during predation bears resemblance to the attachment and junction formation described for merozoite-erythrocyte interactions in the committed phase of invasion following the secretion of microneme and rhoptry contents [5]. Future investigations will examine the attachment process of *Colpodella* sp. to *B. caudatus* for similarities in zoite (invasive stage) attachment to host cells among the apicomplexa and identification of proteins participating in attachment and myzocytosis.

Colpodella species show varied life cycles and the trophozoites vary in size, flagella insertion, prey preferences, length of rostrum and feeding behavior [10]. *Colpodella* cells were observed attached to prey using the apical end of the cell. *Plasmodium falciparum* RhopH3 specific antibody reacted with vesicles in the cytoplasm of *Colpodella* trophozoites. Reactivity of RhopH3 specific antisera with the cytoplasmic vesicles varied among different forms of the trophozoites observed by Giemsa stain and IFA. The vesicle reactivity was unexpected due to the punctate pattern typically observed in merozoite rhoptries following the use of rhoptry specific antibodies in IFA [19,20,23] (Figure 3, panels M-P, Figure S1). It is unclear if the vesicles are rhoptry bodies (bulbs) or trichocysts of *Colpodella*. Antibodies against a *P. yoelii* recombinant RhopH3 protein and isolated rhoptries also reacted with cytoplasmic vesicles in *Colpodella* sp. (results not shown). Immunoelectron microscopy will be performed to identify the distribution of RhopH3 and the localization of the protein within the apical compartment of *Colpodella* trophozoites. Disruption of microtubule function and inhibition of myzocytosis will also be investigated to identify the essential molecules required for predatory activity by *Colpodella* trophozoites. The use of Giemsa for staining diprotist cultures and the specificity of the *P. falciparum* antibodies used for IFA provides a consistent protocol that will permit differentiation of *Colpodella* sp. from *B. caudatus* in culture. Beta tubulin distribution is varied among flagellate protists, in particular among kinetoplastids [26,27]. Cellular distribution of tubulins is dependent on stages of the cell cycle as well as life cycle of the organism [26], with cytoskeletal distribution differences observed among subpellicular and flagellar structures. Differences in tubulin isotype distribution within cells have also been identified, demonstrating that heterogeneity in tubulin distribution is not limited to the types of tubulin [28]. In the current study, anti-β-tubulin antibody (KMX-1) was used in IFA in an attempt to distinguish *B. caudatus* from cells of *Colpodella* sp. However, low reactivity of KMX-1 anti-β-tubulin antibody was observed on the cell body of *B. caudatus*. In contrast, KMX-1 reacted strongly with the cell body of *Colpodella* trophozoites and with flagella (Figure 3, panels A-D). The strong cross reactivity of *P. falciparum* rhoptry protein specific antibody with the cytoplasmic vesicles of *Colpodella* sp. supports our hypothesis that the RhopH3 protein is conserved in *Colpodella* sp. Evaluation of protein conservation in other species of *Colpodella* will be needed to confirm the distribution of RhopH3 and other rhoptry proteins among the ancestral free-living relatives of apicomplexans. Furthermore, generation of antibodies against *Colpodella* sp. proteins will provide additional tools for a more detailed characterization of the *Colpodella* sp. life cycle.

Conclusion

The availability of routine staining protocols for light microscopy and antibodies specific for apical complex proteins in *Colpodella* sp. will be instrumental in investigations to fractionate the apical complex organelles. Isolation of *Plasmodium* merozoite rhoptries and proteomic analysis of the rhoptries was facilitated by the availability of antibodies that could identify rhoptry fractions [29,30]. Our data show for the first time that RhopH3 is conserved in *Colpodella* sp, paving the way for identification of other apical complex proteins and the availability of additional genes to aid phylogenetic investigations. Knowledge of developmental stages in the *Colpodella* life cycle associated with the presence of apical complex organelles, will aid studies aimed at biochemical characterization of trophozoites [31]. *Colpodella* sp. like organisms were identified in a clinical case of relapsing infection [32]. Consistent differentiation and identification of *Colpodella* sp. will be helpful in the future when identification of infecting organisms becomes necessary. Finally, the attack index developed in this study allows quantitation of each protist in culture and allows for

determination of predator-prey ratios in culture. Routine Giemsa staining and IFA assays will greatly facilitate future cell biological, molecular and biochemical investigations of *Colpodella* species.

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