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Voromonas Pontica Identified by Giemsa Staining and Anti-RhopH3 Protein Reactivity

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Abstract

Culture characteristics, routine staining for light microscopy and feeding behavior of *Voromonas pontica* Cavalier-Smith (American Type Culture Collection, ATCC 50640), a predatory free-living marine colpodellid, was investigated. Hay medium in sea water bacterized with *Enterobacter aerogenes* was used to establish a diprotist culture with *Percolomonas cosmopolitus* as prey. We investigated the reactivity of anti-*Plasmodium* rhoptry and RhopH3 antibodies to trophozoites of *V. pontica*, using antibodies previously shown to react with *Colpodella* sp. (ATCC 50594) trophozoites. Formalin and methanol-fixed cells were stained for light and fluorescent microscopy. We show that tubular "cytoplasmic bridges" are prominent in the attachments of *Colpodella* sp. to the plasma membrane of *Bodo caudatus* prey while attachments observed for *V. pontica* to *P. cosmopolitus* were in close contact without the obvious bridges. *Plasmodium* rhoptry and RhopH3 specific antisera reacted with *V. pontica* trophozoite cytoplasm, apical end and with contents secreted into the *P. cosmopolitus* prey during myzocytosis. These data provide additional evidence that the close phylogenetic relationship between the colpodellids and apicomplexans is strengthened by rhoptry protein conservation.

Keyword: Apical Complex Organelles; *Colpodella*; *Plasmodium falciparum*; RhopH3; Rhoptries; Trichocysts; *Voromonas pontica*

Introduction

The free-living biflagellated colpodellid alveolates are phylogenetically closely related to the apicomplexans [1-3]. In previous studies we showed that antibodies against *Plasmodium* whole rhoptries and the 110 kDa high molecular weight rhoptry protein RhopH3, reacted with cytoplasmic vesicles in *Colpodella* sp. trophozoites [4,5]. *Voromonas pontica* Cavalier-Smith (ATCC 50640) is a predatory free-living marine colpodellid that preys on *Percolomonas cosmopolitus*. Transmission electron microscopy shows *V. pontica* (formerly *Colpodella pontica*) is morphologically similar to *Colpodella* species by the presence of rhoptries and micronemes, but differs from them by possessing trichocysts [2,6]. However, molecular and ultrastructural differences led to a change in classification resulting in a new genus *Voromonas* [2]. In order to facilitate biochemical and molecular characterization of *V. pontica*, culture conditions in Hay medium were established and fixation methods were optimized for routine light microscopy. Staining and antibody characterization of *V. pontica* was also performed. In this study we show that *Plasmodium* rhoptry and RhopH3 specific antibodies react with proteins associated with cytoplasmic structures in *V. pontica* trophozoites. Furthermore, proteins present extracellularly in areas surrounding predator-prey attacks also reacted with antibodies, similar to data reported for *Colpodella* sp. [4,5]. These types of studies have not been performed previously with *V. pontica*.

Materials and Methods

Cell Culture

Voromonas pontica Cavalier-Smith (ATCC 50640) and *Colpodella* sp. (ATCC 50594) (American Type Culture Collection, Manassas, Virginia, USA) were cultured with prey species; *Percolomonas cosmopolitus* and *Bodo caudatus*, respectively in diprotist cultures in bacterized Hay medium (Ward's Science, Rochester, New York, USA). Seawater media for *V. pontica* culture was prepared by mixing 1800 ml of Carolina Seawater (Carolina Biological Supply Company, Burlington, North Carolina, USA) with 125 ml of concentrated Hay medium (Ward's Science) without wheat seeds. Thirty milliliters of medium in plug seal capped T25 or T75 tissue culture flasks (surface area of 25 cm^2 or 75 cm^2 respectively), bacterized with a single colony of *Enterobacter aerogenes* cultured on nutrient agar plates (incubated at 37 ^{0}C) and the Hay medium was incubated at 22-24 ^{0}C. *Colpodella* sp. was cultured

as described previously [4,5]. *Voromonas pontica* and *Colpodella* sp. cultures were subcultured every 5 to 7 days by transferring 0.5 ml or 1 ml of scraped cells into 10 ml of bacterized seawater medium or Hay medium, respectively, in 25 cm^2 tissue culture flasks capped tightly with plug-seal caps. Cultures were examined using an inverted microscope with phase contrast and Giemsa staining was performed to monitor cell growth. All cultures were maintained aseptically without antibiotics or filtration for bacteria elimination. *Plasmodium falciparum* was cultured as described previously and schizont-infected erythrocytes harvested and processed for immunofluorescence assay (IFA) as described [7]. Several (>5) batches of *V. pontica* cultures were harvested and fixed for Giemsa staining and IFAs to evaluate consistency and reproducibility of data obtained.

Staining and Light Microscopy

Voromonas pontica diprotist cultures were processed for staining by collecting 3 ml of culture into Eppendorf tubes. Tubes containing a volume of 1.5 ml each was centrifuged at 13,000 xg for 10 min, discarding supernantant and resuspending pellets in 100 µl of 1X PBS. Smears were prepared on glass slides from the cell suspension for methanol fixation, air-dried and either stained with Giemsa stain or incubated with antibodies for IFA. The second aliquot of 1.5 ml was centrifuged as indicated and the pellet was resuspended in 250 µl of 1X PBS. An equal volume of 10% formalin was added to the cell suspension, resuspended gently followed by incubation for 10 min at room temperature (RT). Cells were centrifuged for 10 min at 13,000 xg. The supernatant was discarded and the pellet was washed with 500 µl of 1X PBS and after discarding the supernatant, the formalin fixed cells were resuspended in 80 µl 1X PBS and smears prepared on glass slides for Giemsa staining and IFA using anti-β-tubulin and rhoptry specific antibodies [8-10]. Giemsa staining and IFAs were performed multiple times (>5 times), using different batches of cells, to confirm reproducibility.

Large volumes of diprotist cultures of *V. pontica* and *Percolomonas cosmopolitus* were also harvested and placed in 15 or 50 ml sterile tubes, centrifuged at 1475xg for 10 min, the supernatants were discarded and the pellets resuspended, transferred to Eppendorf tubes and centrifuged at 13000 xg. Before discarding, supernatants were examined for the presence of organisms. For light microscopy, smears were prepared on glass slides from the pellets and 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 in T-flasks (for *Colpodella* sp.) for 10 min at room temperature. Fixed cells in culture flasks were gently scraped and transferred to centrifuge tubes and centrifuged as indicated [5]. Following the removal of supernatant, the pellet was resuspended, transferred to a 1.5 ml eppendorf tube and washed once in 1x PBS and processed for Giemsa staining or IFA as described [4,5]. Cell activity was observed in culture flasks using an inverted microscope and in wet mounts on glass slides. 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 adjusted to 300 dpi using the CMYK color mode on Adobe photo shop (CS6).

Immunofluorescence and Confocal Microscopy

Immunofluorescence and confocal microscopy was performed on *P. falciparum* infected erythrocytes, *Colpodella* sp. and *B. caudatus* diprotist culture and *V. pontica* and *P. cosmopolitus* diprotist cultures as described previously [7] using absolute methanol, acetone or formalin fixed (2% and 5%) cells. Formalin-fixed cells from the diprotist culture were permeabilized with 0.5% Triton X-100. Confocal microscopy was performed at the Cleveland Clinic, Lerner Research Institute Imaging Core, Cleveland, OH, USA. Briefly, smears fixed in cold methanol alone or methanol and acetone combinations for 5 min at -20 °c were incubated with rabbit and mouse polyclonal antibodies or mouse monoclonal antibodies; either individually or together in colocalization experiments. For colocalization assays, mouse antibodies, *Physarium polycephalum* anti-β-tubulin mouse monoclonal antibody KMX-1 [8] (gift from Dr. Bibo Li), 1:1000 dilution and mouse anti-His-FLPbRhop-3 (FL), 1:200 dilution [9], and rabbit primary antibodies; anti-*P. falciparum* recombinant RhopH3 (gene ID PF3D7_0905400) (Plasmodb.org) rabbit antiserum 686 [10] and rabbit anti-*P. falciparum* whole rhoptries, antiserum 676 [12] diluted 1:100 were used as primary antibodies. The secondary antibodies directed to both species, conjugated to different colored Alexa fluorochromes, were used for detection of primary antibodies. Incubation with primary antibodies was carried out as described [4,5] and mounted in Vectashield containing 4′, 6-diamidino-2-phenylindole (DAPI; Vector, Burlingame, CA, USA) or Fluoromount-G (Southern Biotech, Birmingham, AL, USA). Normal (preimmune) mouse or rabbit serum (NMS and NRS, respectively), were used as negative controls for IFA. Images were collected using a Leica TCS-SP5II upright laser scanning confocal microscope (Leica Microsystems, GmbH, Wetzlar, Germany). In addition, an SP8 True Scanning Confocal (TSC) on a DMI8 inverted microscope was used to generate differential interference contrast (DIC) images. 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 x 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.

Results

Giemsa Staining of *V. pontica* and *P. cosmopolitus*

Both *V. pontica* (predator) and *P. cosmopolitus* (prey) were identified in culture (Figure 1, panel A, *V. pontica* and panel B, *P. cosmopolitus*). The three short flagella and one long flagellum were detected in *P. cosmopolitus* (Figure 1B). The biflagellated predator

(panel A) could be distinguished from the tetraflagellate (three short and one long flagella) prey. Cells undergoing myzocytosis were also observed (Figure 1, panels D and E with red arrowheads) showing points of attack between *V. pontica* (top) and *P. cosmopolitus* (below). Formalin fixed cells were identified clearly, compared to methanol fixed cells (Figure 1, panels C and F). *Bodo caudatus* (Figure 1, panel G) was clearly identified from *Colpodella* sp. (panels H and I) by the presence of a prominent kinetoplast. In contrast to the close attachment during myzocytosis in *V. pontica* and *P. cosmopolitus* interactions, a "cytoplasmic bridge" [11] or "tether" (black arrowheads) was identified between *Colpodella* sp. on the left and *B. caudatus* on the right side of the images, during feeding (Figure 1, panels J, K and L).

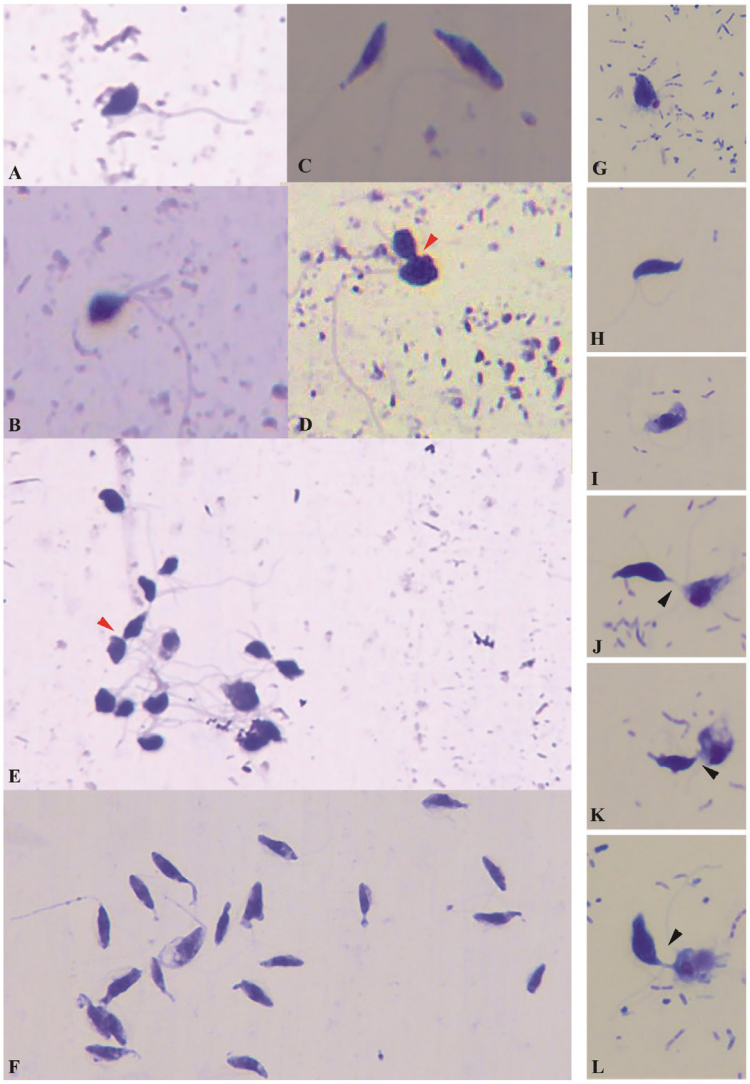

Figure 1: Giemsa stain of diprotist culture containing *V. pontica* and *P. cosmopolitus* and *Colpodella* sp. and *B. caudatus*. **A)** Formalin fixed *V. pontica*; **B)** Tetraflagellate *P. cosmopolites;* **C)** Methanol fixed *V. pontica* and *P. cosmopolitus* cells; **D)** *V. pontica* and *P. cosmopolitus* in myzocytosis. Attached cells (red arrowhead); **E)** *V. pontica* (top) and *P. cosmopolitus* (below); **F)** Methanol fixed *V. pontica* and *P. cosmopolitus* cells; **G)** *B. caudatus* with prominent kinetoplast; **H&I**) *Colpodella* sp. trophozoites; **J,K&L**) *Colpodella* sp. (on the left) interacting with *B. caudatus* (on the right) showing tubular cytoplasmic bridges connecting both cells during myzocytosis. 1,000x magnification

Immunofluorescence and Confocal Microscopy using Anti-Rhoptry and Anti-β-Tubulin Antibodies

Antibodies against *Plasmodium* whole rhoptries and recombinant RhopH3 protein were reacted with methanol and formalin-fixed cells. Anti-*P. falciparum* rhoptry (detected as green) and anti-*P. berghei* RhopH3 (detected as red) were incubated together on smears prepared from a *V. pontica* and *P. cosmopolitus* diprotist culture (Figure 2A, panels i-v). Merged+DIC and DAPI+DIC images (panels i and ii) show *V. pontica* with antibody reactivity in the cytoplasm and nuclear staining with DAPI. Both flagella of *V. pontica* were identified in the DIC image. Anti-rhoptry antibody (detected as green) and anti-β-tubulin antibody (detected as red) were incubated together (Figure 2B, panels i-v). The merged images + DIC and DAPI+DIC showed antibody reactivity with biflagellated cells. Anti-β-tubulin reactivity was prominent on the cell bodies of the trophozoites but distinct from the sites of anti-rhoptry staining in the cell cytoplasm (Figure 2B, panels iii-v). A similar reactivity was observed in Figure 2C, D and E. with anti RhopH3 antibodies and anti-β-tubulin, with apical staining by anti-RhopH3 observed in trophozites (Figure 2B and C, panels iii-v). Positive control antibody reactivity with *P. falciparum* schizonts showed the typical punctate staining pattern observed with anti-RhopH3 staining (Figure 2F) [10]. Negative controls with pre-immune rabbit and mouse serum showed no reactivity with *V.*

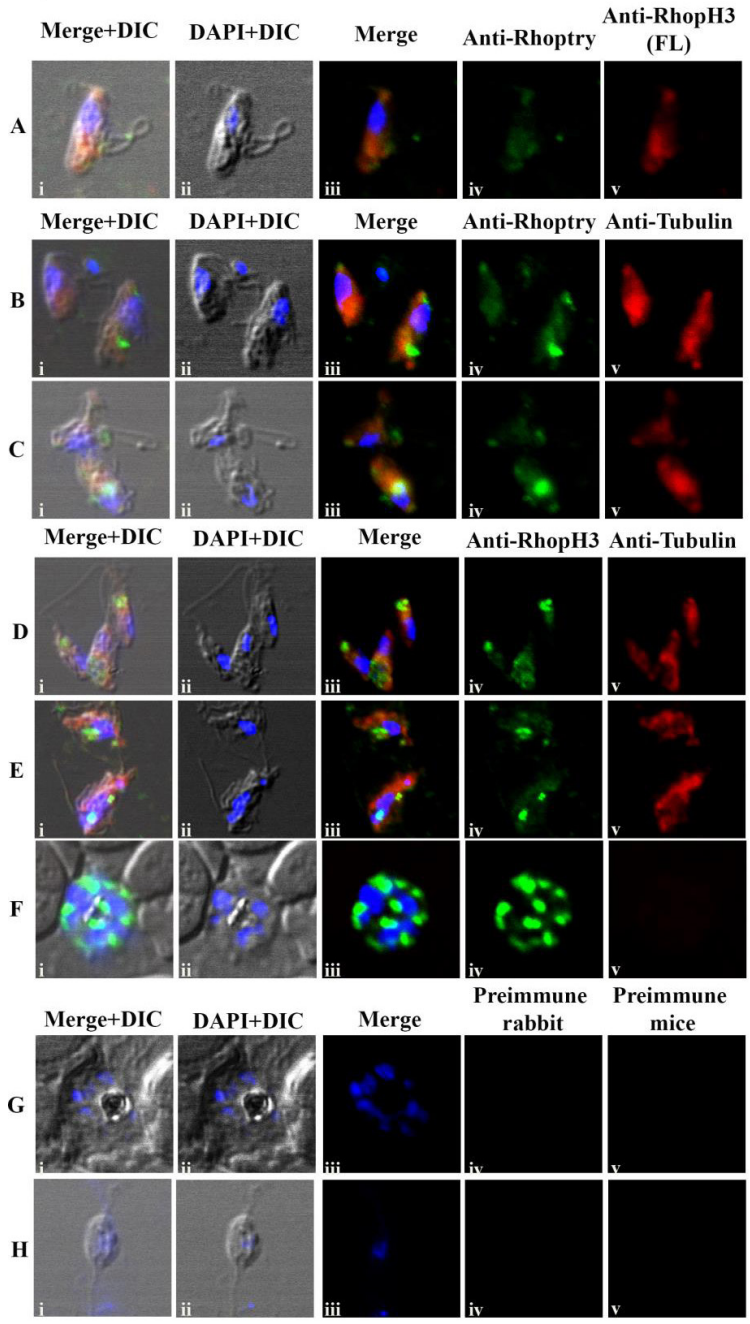

Figure 2: Indirect immunofluorescence assay (IFA) of a diprotist culture containing *V. pontica* and *P. cosmopolitus* and IFA of *Plasmodium falciparum* schizonts. Mouse antiserum FL specific for the high molecular weight rhoptry protein, RhopH3 of *P. berghei* (detected as red) and whole rhoptries of *P. falciparum,* rabbit antiserum 676 (detected as green). **A)** Anti-rhoptry (green) and anti RhopH3 (red) incubated together with smears prepared from *V. pontica* and *P. cosmopolitus* diprotist cultures. Both antibodies reacted with *V. pontica* but not *P. cosmopolitus*; **B)** Rabbit anti-rhoptry (green) and anti-β-tubulin mouse monoclonal antibody (red). Rhoptry and RhopH3 specific antibodies were reactive with *V. pontica*. Merge+DIC (panel i) and DAPI+DIC (panel ii) show three cells, with two cells intensely reactive. A third cell in the middle stained by DAPI was weakly reactive with anti-rhoptry antibodies but not with anti-β-tubulin antibody; **C)** Reactivity of rhoptry and β-tubulin specific antibodies with *V. pontica* was similar to B, D and E. **D&E)** Anti-*P. falciparum* RhopH3 antibody (antiserum 686, detected as green) was reactive with cytoplasmic proteins of *V. pontica* and with the apical end of the trophozoites; **F)** Positive control rabbit anti-*P. falciparum* RhopH3 reactivity with *P. falciparum* rhoptries in segmented schizonts, showed a punctate staining pattern; G. Negative control preimmune (normal) rabbit and mouse serum showed no reactivity with *P. falciparum* schizonts. H. Negative control preimmune rabbit and mouse serum showed no reactivity with *V. pontica*. 3,780x magnification.

pontica and *P. falciparum* (Figure 2,G and H). At points of attack between *Colpodella* sp. and *B. caudatus,* antibody was reactive with proteins associated with structures observed in the cytoplasm of trophozoites and around the points of contact as detected in previous studies [6]. Formalin fixed *Colpodella* sp. shown in attack during myzocytosis with *B. caudatus* are reactive with anti-rhoptry antibody (detected as green) and anti-β-tubulin antibody (detected as red) (Figure 3A and B). *Colpodella* sp. flagella were intensely reactive with anti-β-tubulin antibodies. Similarly, reactivity of anti-RhopH3 antibody was observed with two *Colpodella* sp. feeding stages (Figure 3B, panels i-v). DAPI stained nuclei of prey were observed along with RhopH3 antibody stained (detected as green) structures. In smears prepared from actively feeding diprotist cultures of *V. pontica* and *P. cosmopolitus*, anti-rhoptry and anti-RhopH3 antibody reactivity was observed on trophozoites and on proteins in the vicinity of prey attack (Figure 4). Several

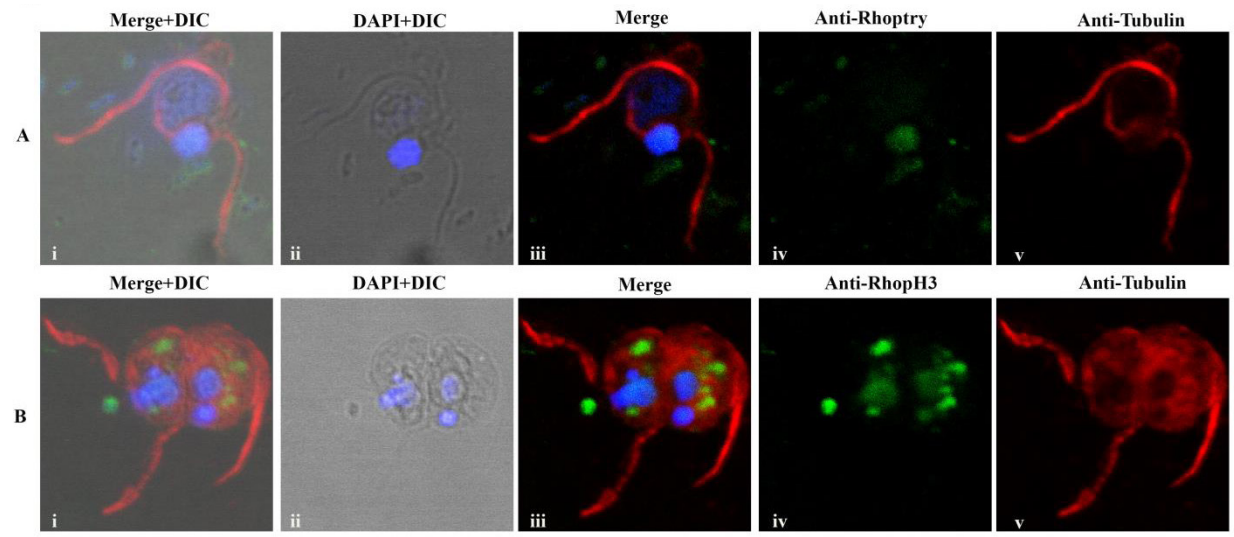

Figure 3: Immunofluorescence and confocal microscopy of a diprotist culture containing *Colpodella* sp. and *B. caudatus*. Formalin fixed and triton permeabilized cells were reacted with rabbit anti-RhopH3 and anti-β-tubulin mouse monoclonal antibodies. **A)** *Colpodella* sp. at end stages of attack on *B. caudatus*. **B)** Two *Colpodella* sp. in attack with *B. caudatus* showing vesicles (detected as green) reactive with anti-RhopH3 antibody. Cell bodies and flagella were reactive with anti-β-tubulin antibody. 3,780x magnification.

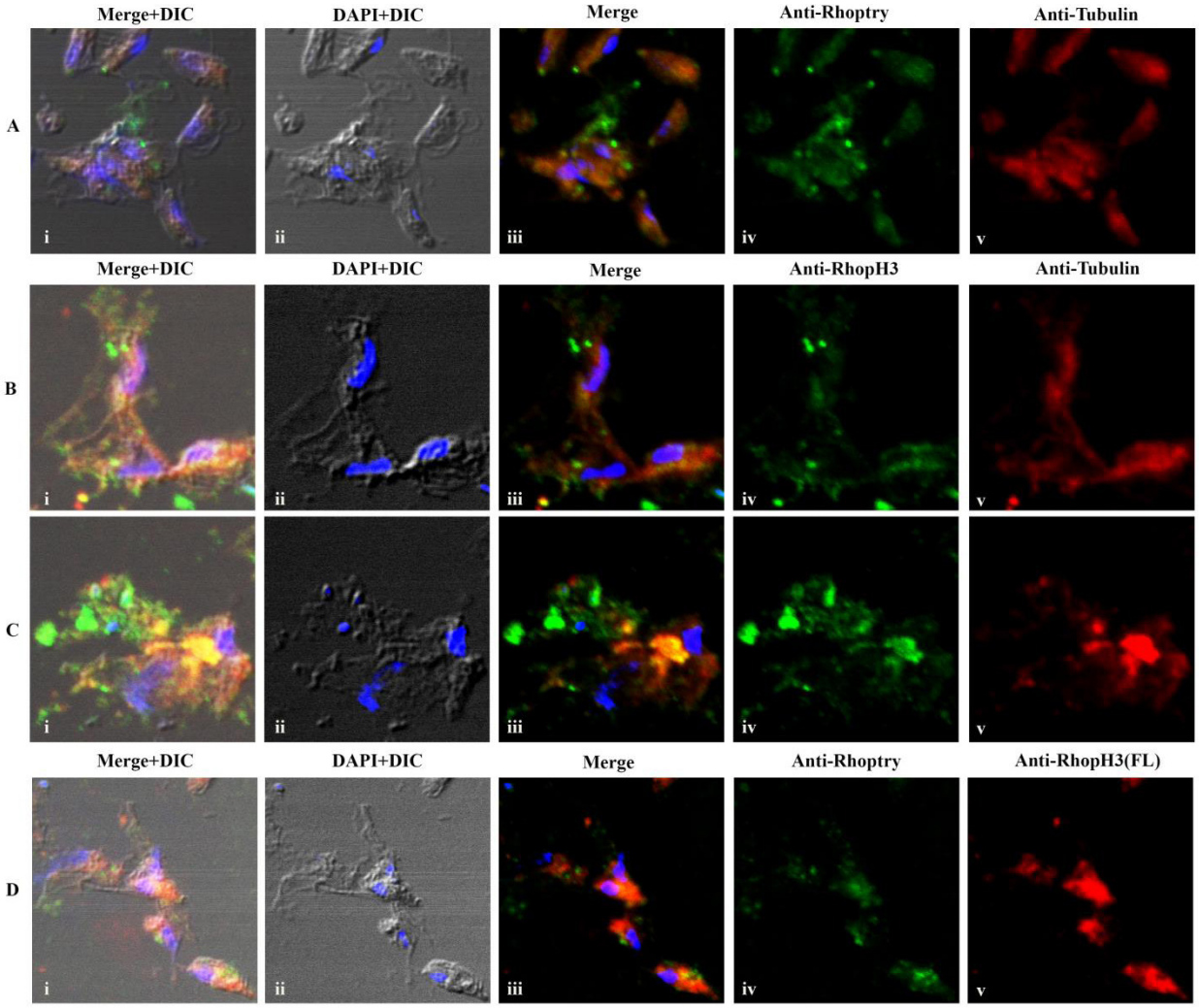

Figure 4: Immunofluorescence and confocal microscopy of actively feeding culture of *V. pontica* and *P. cosmopolitus*. **A)** Merge+DIC showed several cells interacting. Rhoptry specific antibodies (detected as green) were reactive with several foci within cells and in areas surrounding attacks; **B&C)** Cells feeding showed anti-RhopH3 reactive cells and extracellular structures containing protein reactive with antibody. Multiple interactions were observed (in C) with intense rabbit anti-RhopH3 reactivity observed (detected as green) not colocalized with anti-β-tubulin reactivity; **D)** Individual *V. pontica* trophozoites were observed reactive with rabbit anti-rhoptry and anti-β-tubulin mouse monoclonal antibody. 3,780x magnification.

cells were observed interacting with antibody reactive foci (Figure 4A, B, C and D). Tetraflagellate *P. cosmopolitus* trophozoites were not reactive with rhoptry and RhopH3 specific antibody but reacted weakly with anti-β-tubulin antibody (Figure 4A). Anti-RhopH3 (detected as green) reactivity was prominent in areas surrounding actively feeding cells (Figure 4C). Two foci of anti-RhopH3 and anti-β-tubulin reactivity were observed (Figure 4B and C, panels i and iii). Anti-rhoptry and anti-RhopH3 antibody reactivity was colocalized to the same sites in trophozoites of *V. pontica* (Figure 4D, panels i and iii). The staining and IFA results shown were obtained from evaluating several (>5) batches of *V. pontica* cultures for consistency and reproducibility of methods and data obtained.

Discussion

The aims of this study were to establish culture conditions for a diprotist culture containing the predatory free-living marine alveolate, *V. pontica* and its prey *P. cosmopolitus* using Hay medium in sea water. We also wanted to routinely differentiate both cells in culture by light microscopy, using Giemsa staining. Following the routine maintenance of cells in the diprotist culture, we characterized the cells using antibodies specific to *Plasmodium* whole rhoptries and the 110 kDa high molecular weight rhoptry protein, RhopH3. *Voromonas pontica* diprotist cultures were established and maintained in Hay medium prepared with sea water. Both predator and prey encysted in culture, allowing for subcultures to be performed within 5 to 7 days. Cells obtained from cultures were fixed for staining and cell biological analyses. With Giemsa staining, trophozoites of both predator and prey could be differentiated, based on morphology and particularly, by the differences in the number of flagella; two flagella identified in *V. pontica* and four in *P. cosmopolitus.* Predator-prey attacks were also detected by Giemsa staining showing close contact of *V. pontica* to its prey. We investigated the reactivity of antibodies specific to *P. falciparum* rhoptries and a 110 kDa rhoptry protein, RhopH3. Cross-reactivity of antibodies with proteins of *V. pontica* was observed. Additional studies will confirm the identity of the proteins and the structures associated with antibody reactivity. In previous studies, we showed that *Plasmodium* rhoptry and RhopH3 specific antibodies reacted with "doughnut shaped" vesicles in the cytoplasm and apical ends of *Colpodella* sp. (ATTC 50594) trophozoites [4,5]. Due to the close phylogenetic relationship between *V. pontica* and *Colpodella* species, and between the colpodellids and apicomplexans [1-3], we tested the hypothesis that *V. pontica* shares conservation of the RhopH3 encoded protein with *Colpodella* sp. (ATTC 50594) and *Plasmodium* species. Rhoptry proteins have not been identified previously in *V. pontica*. Furthermore, routine staining for light microscopy using Giemsa stain has not been performed to differentiate *V. pontica* from its prey protist.

In this study we show that the RhopH3 specific antibodies recognize cytoplasmic structures in trophozoites of *V. pontica*. Reactivity of antibodies to whole rhoptries of *P. falciparum* with *V. pontica* trophozoites, suggests additional rhoptry proteins may be shared between *Plasmodium* species and *V. pontica*. Future studies will confirm the distribution of antibody reactive proteins in *V. pontica*. Morphologically and ultrastructurally, *V. pontica* is similar to dinoflagellates due to the presence of trichocysts in the trophozoites, differences in organization of cortical alveoli and the presence of a non-photosynthetic plastid [2,11,12]. Additional differences between *V. pontica* and *Colpodella* species were observed in *C. vorax* and *C. tetrahymena* where a diminished rostrum is present in *V. pontica* compared to the *Colpodella* species [2,11]. We observed some of these differences by Giemsa staining; there was close contact between *V. pontica* and *P. cosmopolitus* during predator-prey attacks (Figure 1, panels D and E). The close contact, presumably due to the diminished rostrum was a major difference observed when compared to the distinct tubular cytoplasmic bridges or tethers connecting *Colpodella* sp. and *B. caudatus* during predator attack (Figure 1, panels J,K and L). Multiple predator attachments were common with *Colpodella* sp. interacting with *B. caudatus* [4] while single prey attachments were predominant with *V. pontica* interacting with *P. cosmopolitus* in Hay medium. The stage of predation, whether early or late in the attack, may influence how close and tightly the predator is attached to the plasma membrane of the prey. In later stages when the predator has increased in size from aspirating prey contents, the point of attack may be tight against the prey's membrane. Cavalier-Smith and Chao [2] reported a closer phylogenetic relatedness of the ectoparasitic *C. tetrahymena* to free-living *Colpodella* sp. (ATCC 50594), in contrast to the divergence observed between *C. tetrahymena* and *V. pontica* [2]. These differences led us to question whether *V. pontica* expressed RhopH3, a protein that is involved in nutrient acquisition in *P. falciparum* [13]. *Voromonas pontica* shares the presence of rhoptries and micronemes with *C. angusta, C. edax, C. perforans* and *C. vorax* [2,11] and the presence of rhoptries with *C. gonderi* and *C. pugnax* [2,11]. Both bulbous (pear-like) and clavate rhoptries have been described among colpodellids, with *V. pontica* sharing clavate structure of the rhoptries with *C. pugnax, C. perforans* and *C. edax* [11]. *Plasmodium* merozoite rhoptries are bulb or pear-shaped [9,10]. The organization of a microtubular band located inside the pseudoconoid in *V. pontica* and *C. edax* was reported to be also similar [2,11]. Following contact with its prey, the pseudoconoid of *V. pontica* penetrates the plasma membrane in a manner similar to the attachment and penetration of *Colpodella* sp. to its prey, and attachment of invasive zoite stages to host cells among apicomplexans and use of the conoid in parasitic *Perkinsus* for attachment and penetration of the bivalve mollusk host cell [11,14]. Antibodies against whole rhoptries of *P. falciparum* and RhopH3 reacted with proteins associated with cytoplasmic structures of *V. pontica* trophozoites. However, cytoplasmic vesicles identified in *Colpodella* sp. were not observed in *V. pontica* [4,5]. Although antibody reactivity was observed at the apical end of *V. pontica* trophozoites, it is unclear if the reactivity was associated with rhoptries or trichocysts. In addition to antibody reactivity with proteins inserted into the prey during penetration of the plasma membrane, antibodies also reacted with proteins extracellularly, at the point of contact and in the areas surrounding predator attack (Figure 4). Future investigations will be required to determine the cellular distribution of proteins identified by *Plasmodium* rhoptry and RhopH3 specific antibodies in *V. pontica*. Additionally, availability of antibodies against *V. pontica* apical complex proteins will be useful in defining the organization, biogenesis and function of apical complex organelles in *V. pontica* and in explaining the mechanisms of myzocytosis in *V. pontica*.

Conclusion

We report for the first time, identification of the RhopH3 rhoptry protein in the predatory marine colpodellid, *Voromonas pontica.* Antibodies specific for whole rhoptries of *P. falciparum* and recombinant RhopH3 of *P. falciparum* and *P. berghei* reacted with proteins associated with cytoplasmic structures of *V. pontica*. The presence of four flagella in *P. cosmopolitus*; three short and one long flagella were used for differentiation of the biflagellated *V. pontica* from its prey [15,16]. Although possessing divergent morphological features that necessitated separation from the genus *Colpodella* [2], *V. pontica* uses its pseudoconoid contained within a rostrum to attack and penetrate the prey's cell membrane, after which the cytoplasmic contents of the prey are aspirated [11]. Single predator-prey attachments were commonly observed in Hay medium culture by phase contrast microscopy, in contrast to *Colpodella* sp. diprotist cultures where up to four predators could be observed attacking a single *B. caudatus* prey [4]. Rhoptry specific antibody reactivity was observed both within the cytoplasm of *V. pontica* trophozoites and in the areas surrounding predator-prey attacks. Although it is unclear whether rhoptry protein distribution is associated with rhoptries, trichocysts or a different organelle, the conservation of RhopH3 is shared by *Plasmodium* species, *Colpodella* sp. (ATCC 50594) and *V. pontica* (50640) in agreement with transcriptomic evidence for shared genes between colpodellids, chromerids and apicomplexans [17]. Routine culture in Hay medium and staining of cells in culture will aid ultrastructural, biochemical and molecular analyses of *V. pontica* and facilitate investigations of the origins of intracellular parasitism among apicomplexans. Rhoptry genes may aid phylogenetic analyses among colpodellids and apicomplexans.

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