LOCALIZATION OF THE CYSTOGENOUS GLANDS OF *OPISTHORCHIS VIVERRINI* CERCARIAE

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Abstract. Opisthorchiasis in northeastern Thailand is an important etiology of cholangiocarcinoma. To form the infectious stage, free swimming cercariae penetrate cyprinid fish, shed their tails, and then secret a cystic substance to cover their larval stage to form metacercariae in the fish body. We determined the location of the cystogenous glands in *Opisthorchis viverrini* cercariae. The cercariae and metacercariae were obtained from the naturally infected snail host, *Bithynia siamensis goniomphalos* and from cyprinid fish, respectively. The cyst walls of the metacercariae were separated and used to immunize inbred male BALB/c mice to obtain cyst wall antibodies. The general characteristics of the *O. viverrini* cercariae and metacercariae were studied by hematoxylin and eosin (H&E) staining of sections. The location and ultrastructure of the cystogenous glands of cercariae were studied by immunoperoxidase, immunofluorescence and transmission electron microscopy. The structures and organelles of cercariae and metacercariae could be identified, but the cystogenous glands could not be detected in H&E sections. The immunoperoxidase and immunofluorescence sections revealed positive reactions for cystogenous glands predominanted in the lateral part of the cercariae and were clearly seen in the cyst wall of the metacercariae. The ultrastructure of the cystogenous glands contained semitranslucent electron dense oval shaped granules. If interference occurs during the formation of the cysts by fish immune response, the metacercariae may not develop to maturity. It may be easily digested or degraded by human stomach acid and pepsin. This may be an efficient method for control of *O. viverrini* infection which requires further detailed study.

Keywords: *Opisthorchis viverrini,* cercariae, cystogenous gland, immunoperoxidase, immunofluorescence, transmission electron microscopy

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INTRODUCTION

Human liver fluke infection, opisthorchiasis and subsequent cholangiocarcinoma (CCA), are caused by *Opisthorchis viverrini* (Southeast Asia), *Opisthorchis felineus* (Eastern Europe and the Far East), and *Clonorchis sinensis* (China, Taiwan,

northern Vietnam, and Korea). *O. viverrini* is endemic in Thailand, Lao PDR, Cambodia, and southern Vietnam (WHO, 1995; IARC, 2011). It causes pathology of the hepatobiliary system, including cholangitis, obstructive jaundice, hepatomegaly, cholecystitis, and biliary lithiasis. Epidemiological evidence and experimental studies have shown *O. viverrini* infection is an etiological factor of CCA with the highest incidence found in northeast Thailand (Thamavit *et al*, 1978; Harinasuta *et al*, 1984; Haswell-Elkins *et al*, 1992; IARC, 1994; Sitithaworn *et al*, 1994; Vatanasapt *et al*, 2000; Watanapa and Watanapa, 2002; Mairiang and Mairiang, 2003; Honjo *et al*, 2005). In Thailand, an estimated 6 million people are infected with *O. viverrini* (Jongsuksuntigul and Imsomboon, 2003).

The prevalence of *O. viverrini* infection in Thailand was found to be 24.5%, and higher in men (27.6%) than in women (21.4%) (Sriamporn *et al*, 2004). Humans become infected with *O. viverrini* by eating fermented, uncooked or partially cooked cyprinid fish containing infective metacercariae (Sadun, 1955; Harinasuta and Vajrasthira, 1960). The life cycle of *O. viverrini* requires two intermediate hosts: bithyniid snails as first and cyprinid fishes as second intermediate hosts, while dogs and cats serve as reservoir hosts (Ditrich *et al*, 1990; Giboda *et al*, 1991; Scholz *et al*, 2003; Enes *et al*, 2010; Aunpromma *et al*, 2012). The process of forming metacercariae in cyprinid fish (*eg*, *Cyclocheilichthys* spp, *Puntius* spp, *Hampala dispar*) commences with the attachment of cercariae on the surface of the fish, with subsequent tail shedding and penetration into the fish tissues. The cercariae encyst as metacercariae mainly in the head and body muscle of fish (Harinasuta *et al*, 1961; Wykoff *et al*, 1965; Tansurat, 1971; Viranuvatti and Stitnimankarn, 1972; Vichasri *et al*, 1982;

Tesana *et al*, 1985).

The cyst wall of the trematode metacercariae is generated from cystogenous glands in the body portion of cercariae. When the encystations of the metacercariae are localized in the tissues of a second intermediate host, there is likely to be a host cellular response resulting in the production of a connective tissue capsule around the cyst wall of the parasite (Schell, 1970). In the process of forming cysts from the cercariae to the metacercariae of the trematode, typically the inner cyst wall forms from the cystogenous glands of the cercariae (Žďárská and Našincová, 1985; Žďárská, 1989; Harada and Suguri, 2001). This is similar to precursors in the layers of *Fasciola hepatica* metacercariae which are synthesized in distinct kinds of cystogenic cells in cercariae. These cells produce protein precursors similar to the cytoplasmic structures of secretory cells. The formation process can be seen in the synthesizing cells during the early development of cercariae (Dixon, 1966; Mercer and Dixon, 1967). Harada and Suguri (2001) found 4 layers in the cyst wall of metacercariae in *Cercaria shikokuensis* which are derived from distinct cell groups of cercariae secretory gland cells.

The present study determined the location of the cystogenous glands, including the shape and size of cystogenous granules in *O. viverrini* cercariae using immunoperoxidase, immunofluorescence and transmission electron microscopy.

MATERIALS AND METHODS

Sample preparation

The *O. viverrini* cercariae were obtained from naturally infected *Bithynia siamensis goniomphalos* snails. The snails were collected from reservoirs of clear, stagnant or slow running water such as

rice fields, ponds, and ditches in Khon Kaen Province, northeastern Thailand. The collected snails were carried back to the laboratory in plastic bags and then cleaned with tap-water. *B. siamensis goniomphalos* snails were identified using published keys (Brandt, 1974; Chitramvong, 1992). Infection with *O. viverrini* was examined using the cercarial shedding method.

The *O. viverrini* cercariae were identified based on their morphology having a tobacco-pipe form when resting on the bottom surface of the container or briefly hanging in the water (Wykoff *et al*, 1965). Confirmation that the cercariae were *O. viverrini*, was done using specific polymerase chain reaction (PCR) primers of OV-6F (5'-CTGAATCTCTCGTTTGTTCA-3') and OV-6R (5'-GTTCCAGGTGAGTCTCTCTA-3') (Wongratanacheewin *et al*, 2001). The identified cercariae were collected, washed with distilled water (DW) several times, and fixed in 10% neutral formalin overnight (Harada and Suguri, 2001; Huffman *et al*, 2009) or Karnovsky's fixative for further study.

The *O. viverrini* metacercariae were obtained from naturally infected cyprinid fish using a pepsin digestion method (Tesana *et al*, 1985). Thereafter, metacercariae were excysted by pressing with a cover slip onto a glass slide. The cyst walls and newly excysted juveniles were individually collected. The cyst walls were placed in a 0.1 M phosphate buffer solution (0.1 M PBS) and stored at -80°C until used. The excysted larvae were washed several times with 0.1 M PBS and fixed in 10% neutral formalin overnight. Mature *O. viverrini* metacercariae were dissected from naturally infected cyprinid fish and from experimental infected fish 10 days post-infections. The morphology of metacercariae was the same as description of Vajrasthira *et al* (1961). The fish tissue containing metacercariae was cut into small pieces then fixed in 10% neutral formalin.

Light microscopic study of cercariae

Fifty cercariae were relaxed and fixed in hot 10% neutral formalin. The body lengths of the cercariae were measured from the anterior margin to the end of the tail. Two portions of body and tail were measured for length and width (at the widest portion). The diameters of the oral sucker, ventral sucker, and excretory bladder were also measured. The length of the tail was measured from the junction to the body toward the end of the tail. The ventral fin-fold which is the nearest to the fore tail was measured from the base of the tail margin to the ventral fin-fold margin and to the dorsal fin-fold near the posterior portion of the tail. The total number of tail nuclei was counted. The aforementioned structures of the cercariae were measured using the DP2-BSW program (Olympus, Tokyo, Japan) under a light microscope, OLYMPUS BX51 (Olympus, Tokyo, Japan).

The cercariae fixed in 10% neutral formalin were dropped onto a slide coated with 0.1% poly-L-lysine and stained with hematoxylin and eosin (H&E) to study the internal organs and their properties. Thereafter, samples were dehydrated with a series of graded ethanol, then mounted on a slide with permount media, and examined by light microscopy. The *O. viverrini* cercariae, metacercariae, and newly excysted juveniles were fixed in 10% neutral formalin and embedded in paraffin. The paraffin blocks were sectioned at 5 m thicknesses using a microtome, stained with H&E, and examined under a light microscope.

Immunolocalization

The cyst walls from above procedure

were homogenized and sonicated at 4°C in 0.1 M PBS which contained protease inhibitor cocktail I (A.G. Scientific, San Diego, CA) at a ratio of 1:100 (by volume), until the solution become homogeneous. The homogenate was centrifuged at 12,000 rpm at 4°C for 30 minutes. The supernatant was transferred in small aliquot into microtubes and stored at -20°C until used.

Inbred male BALB/c mice were subcutaneously inoculated with crude antigen (100 g/mouse) and mixed with Freund adjuvant (Sigma-Aldrich, St Louis, MO) (antigen:adjuvant=1:1 by volume), boostered twice, once a week, and serum was collected after 2 weeks of the last immunization. Serum was kept in a small aliquot in microtubes and stored at -20°C until used for immunolocalization. Animal experimentation in this study was approved by the Animal Ethics Committee of the Faculty of Medicine, Khon Kaen University, Thailand (Ethical Clearance No AEKKU 3/2555).

Immunoperoxidase staining was performed according to the manufacturer's instructions [BioModule™ Immunohistochemical (IHC) Staining for Tissues; (Invitrogen, Carlsbad, CA)]. In brief, paraffin sections of cercariae, metacercariae, and newly excysted juveniles were deparaffinized with xylene. Antigen was retrieved by incubating sections with citrate buffer (pH 6.0) at 110°C for 5 minutes, then eliminating endogenous peroxidase activity with 100 l Peroxo-Block™ for 2 minutes. Non-specific binding was blocked with 5% skim milk for 1 hour. Subsequently, 100 l of the appropriately diluted primary antibody (dilution 1:200 in 0.1 M PBS) was added to sample and unimmunized serum was used for the control group. Two drops of secondary antibody (EnVision+ System-HP labeled Polymer Anti-mouse; Invitrogen, Carlsbad, CA)

were added onto slides and the slides were incubated at room temperature for 1 hour in a moist chamber, visualized with diluted 3,3′-diaminobenzidine (DAB) for 45 seconds, counterstained with hematoxylin, mounted with Histomount™, and the samples were observed with light microscopy.

For fluorescence staining, Alexa 488-labeled goat anti-mouse IgG (Invitrogen, Carlsbad, CA) was used as a marker for secondary antibody. Alexa 488 labeled goat anti-mouse (100 l dilution 1:400 in 0.1 M PBS) was added for 1 hour, washed with 0.1 M PBS containing 0.05% Tween-20 for 3 minutes twice, and mounted with glycerol dilution (1:4 of glycerol and 0.1 M PBS) (Charoensuk *et al*, 2011). Samples were analyzed using the fluorescence microscope (Carl Zeiss, Jena, Germany).

Transmission electron microscopic study

The cercariae were fixed with Karnovsky's fixative (a combination of 3% glutaraldehyde and 4% paraformaldehyde) overnight and post-fixed with 1% osmium tetraoxide for 2 hours. The samples were washed twice with 0.1 M PBS 15 minutes each time, dehydrated in ascending concentrations of ethanol for 10 minutes each concentration, and the propylene oxide was added for 10 minutes. The samples were infiltrated with embedding plastic [a combination of 15 g Araldite 502 (Electron Microscope Polysciences, Washington, PA) 11.5 g DDSA (Polysciences, Warrington, PA), 21 drops catalyst DMP-30 (Fluka Chemie, Buchs, Switzerland)] and mixed with propylene oxide solution in proportions of 1:3, 1:1, and 3:1 for 1 hour each, then embeded in pure plastic overnight. Subsequently, the samples were embedded in a flat mold and allowed to polymerize at 60°C for 48 hours. Samples were sectioned at 75 nm

using an ultramicrotome (RMC, Tucson, AZ) and mounted onto copper grids. Sections were visualized by staining with uranyl acetate and lead citrate for 20 minutes and 10 minutes, respectively. Finally, the sections were viewed under a transmission electron microscope (JEOL, Tokyo, Japan), with a monitor operated Digital Micrograph (Gatan, Pleasanton, CA), and photographs were taken with digital camera. All measurements were taken and recorded. Mean and standard deviations of the measurements of the internal organs were calculated for whole cercariae, head, tails, and granules of the cystogenous glands.

RESULTS

Fifty free swimming cercariae were measured. The average body length was 176.91±14.58 m (mean±SD) and width was 68.46±11.61 m. The length of the whole cercariae were 580.27±32.47 m. The average sizes of the oral sucker (diameter), ventral sucker (diameter), excretory bladder (diameter), ventral fin-folds, dorsal fin-folds, tails, and number of nuclei of tail were 404.50±27.63 m, 29.77±2.87 m, 21.99±2.62 m, 11.31±1.43 m in width, 19.22±1.67 m in width, and 37.66±3.48, respectively. The *O. viverrini* cercariae were morphologically identified, they had scattered brownish pigment, prominent oral sucker, ventral sucker, a pair of eye spots, penetration glands, a tail, and dorsal-ventral fin-folds (Fig 1A, B). Whole cercariae were stained with H&E, which showed oral-ventral suckers, a pair of eye spots, penetration glands, a tail, nuclei of the tail, and fin-folds but the cystogenous glands could not be differentiated (Fig 1C, D). The PCR product size of the cercariae was 330 base pairs, which confirmed morphological identification.
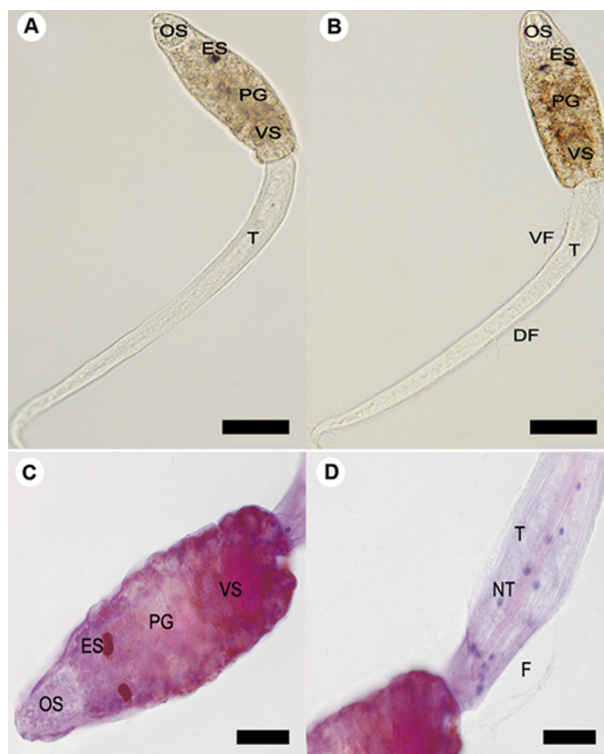

Fig 1–The *O. viverrini* cercariae. Cercariae were fixed with 10% neutral formalin and photographed under a light microscope equipped with a digital camera, using the DP2-BSW program. A) cercaria in the vertical position; B) horizontal position. C, D) the body of the cercaria appears as brownish scattered pigmentation; whole *O. viverrini* cercaria stained with H&E showing the body portion; C) and the tail portion, (D). OS, oral sucker; ES, eye spot; PG, penetration gland; VS, ventral sucker; T, tail; NT, nucleus of the tail; VF, ventral fin-fold; DF, dorsal fin-fold; F, fin-fold. Scale bars=50 m (A, B) and 10 m (C, D).

Cercariae sections were stained with H&E which better revealed the structures than staining of whole cercariae, namely, the oral-ventral suckers, eye spots, penetration glands, a tail, and nuclei of the tail. Penetration glands were presented
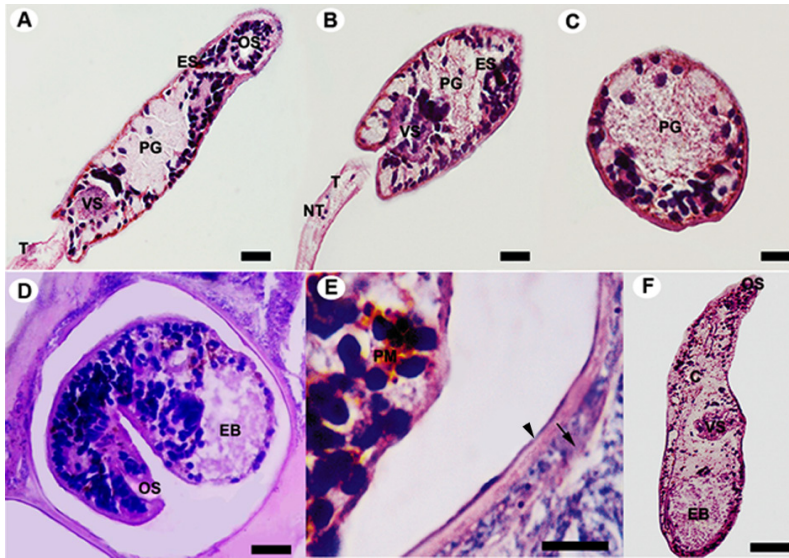
Fig 2–The *O. viverrini* cercaria, metacercaria, and newly excysted juvenile sections stained with H&E. The cystogenous glands in this stage are unidentified. A) sagittal section; B) lateral section; **C**) cross section. D, E) mature metacercaria from naturally infected cyprinid fish sectioned and stained with H&E. E) faint staining of the cyst wall of a metacercaria (arrowhead); connective tissue (arrow) of infected fish; F) section of newly excysted juvenile stained with H&E. OS, oral sucker; ES, eye spot; PG, penetration gland; VS, ventral sucker; T, tail; NT, nucleus of tail; PM, brownish pigment; EB, excretory bladder; C, ceca. Scale bar=20 m (D, F) and 10 m (A-C, E).

control groups (Fig 3E, F). Metacercaria at 10 days post-infection in fish showed strongly positive reactions in the larval body but weak reactions occurred in the cyst wall (Fig 3C). The positive reactions of the mature metacercaria were mostly limited in the cyst wall but there were weak reactions in the larval body (Fig 3D). The positive reactions in the newly excysted juvenile were distributed around the edge of body (Fig 3I). However, non-specific reactions were seen in the bodies of all control groups (Fig 3G, H, J). The positive immunofluorescence reactions occurred mostly at the lateral body of cercariae (Fig 4A-C) and none of the control groups showed a positive reaction (Fig 4D-F). Positive reactions in metacercaria at 10 days post-infection occurred strongly in the larval body but weakly in the cyst wall of metacercaria (Fig 4G). Strongly positive reactions in the mature metacercaria occurred in the cyst wall but weakly in the larval body (Fig 4H). Cross reactions in the control groups were observed (Fig 4I, J). In the newly excysted juvenile, positive reactions were distributed around the edge of body (Fig 4K). The control group showed cross reactions (Fig 4L).
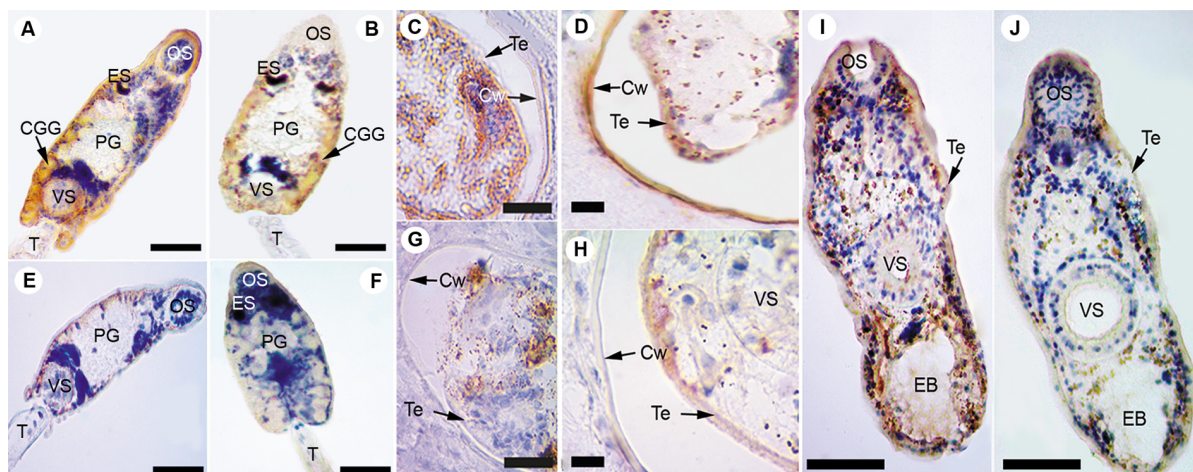
as 5 pairs but the cystogenous glands could not be identified (Fig 2A-C). Following staining of mature metacercariae with H&E, the cyst wall was faintly seen adjacent to the fibroblast tissue of the host, the oral sucker, excretory bladder, and brownish pigment were also seen (Fig 2D, E). Sections of newly excysted juveniles showed oral-ventral suckers, a ceca, and an excretory bladder (Fig 2F).

Localization of the cystogenous glands of cercariae were demonstrated by immunoperoxidase and immuno-fluorescence staining. Immunoperoxidase positive reactions were located mostly on the lateral sides of cercariae (Fig 3A, B). No positive reactions were seen in the

Fig 3–Immunoperoxidase staining of *O. viverrini* cercariae, metacercariae, and newly excysted juveniles. A-D, I) test groups staining with immunized serum. A) Sagittal section of a cercaria in the body portion with evidence of positive reactions bilaterally. B) lateral section presented bilaterally; C) a metacercaria of 10 days post-infection showing positive reaction in the cyst wall and located in the larval body with brown pigments; D) a mature metacercaria showing strong positive reactions in the cyst wall and weak positive reactions in the larval body; I) the positive reactions distributed mostly around the edge of the body of newly excysted juveniles. E, F) control groups of cercariae stained with unimmunized serum showing no positive reaction in the body; G, H, I) control groups of metacercariae at 10 days post-infection, a mature metacercaria, and a newly excysted juvenile, respectively, stained with unimmunized serum showing clumped brown pigments in the body with no positive reaction in the cyst wall of the metacercaria. OS, oral sucker; ES, eye spot; CGG, cystogenous gland; PG, penetration gland; VS, ventral sucker; T, tail; Cw, cyst wall; Te, tegument; EB, excretory bladder. Scale bars=20 m (A, B, E, F, I, J) and 10 m (C, D, G, H).

The ultrastructure of the cystogenous glands of cercariae were seen as many semitranslucent electron dense granules which were oval in shape and located in the dorsolateral area of the cercariae. The mean size of cystogenous granules was 80.40±17.07 nm in length and 50.23±11.16 nm in width. The tegument of the cercariae contained many dense spherical granules and light spherical granules were found embedded and distributed in the tegument. Muscle conjugated from the tegument and secretory granules were detected as electron dense and oval shaped. These granules were secreted from mucoid glands located between the cystogenous glands (Fig 5).

DISCUSSION

The structural measurements of the 50 free swimming *O. viverrini* cercariae were similar in size to previous studies (Wykoff *et al*, 1965; Adam *et al*, 1993). However, lengths of the body and the whole cercariae were longer but the width was shorter than the average size reported by Wykoff *et al* (1965). The length of the body was longer than the length of whole cercariae and the width of the body was
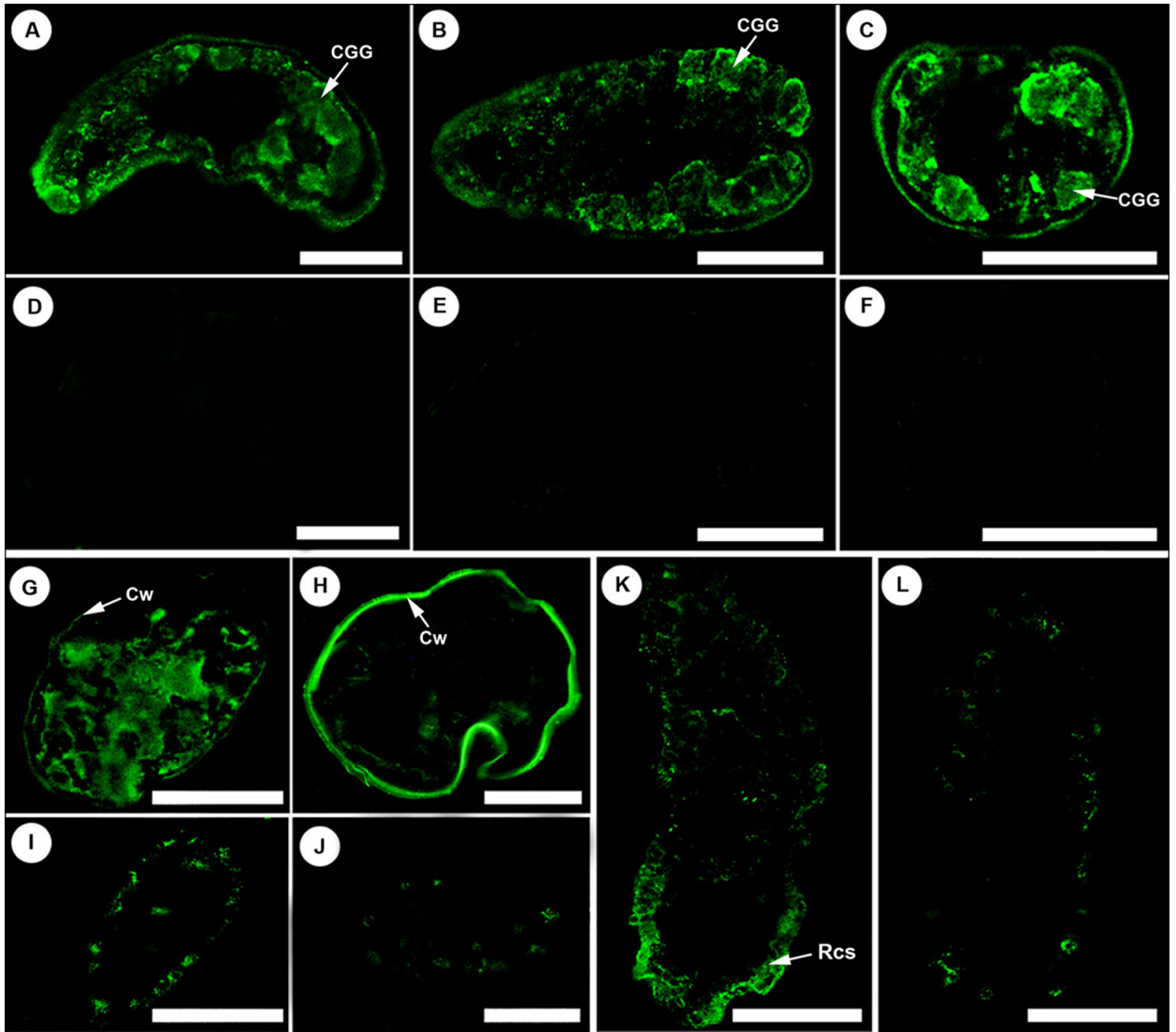
Fig 4–Immumofluorescence staining of *O. viverrini* cercariae, metacercariae, and newly excysted juveniles. A-C, G, H, K) Stained with immunized serum. A-C) A positive reaction presented at the lateral part of the body of a cercaria. A) Sagittal section; B) lateral section; C) cross section. D-F) Sections of cercariae stained with unimmunized serum (control group) with no positive reaction. G) A metacercaria at 10 days post-infection showing weak positive reactions in the cyst wall and strong positive reactions in the larval body; H) A strongly positive reaction in the cyst wall of a mature metacercaria and a weak positive reaction in the larval body; K) A positive reaction in a newly excysted juvenile distributed mostly around the edge of body. I, J, L) A metacercaria at 10 days post-infection, a mature metacercaria, and a newly excysted juvenile, stained with unimmunized serum (control group) showing cross reactions. CGG, cystogenous gland; Cw, cyst wall; Rcs, remaining cystogenous substance. Scale bars=20 m (A-L).
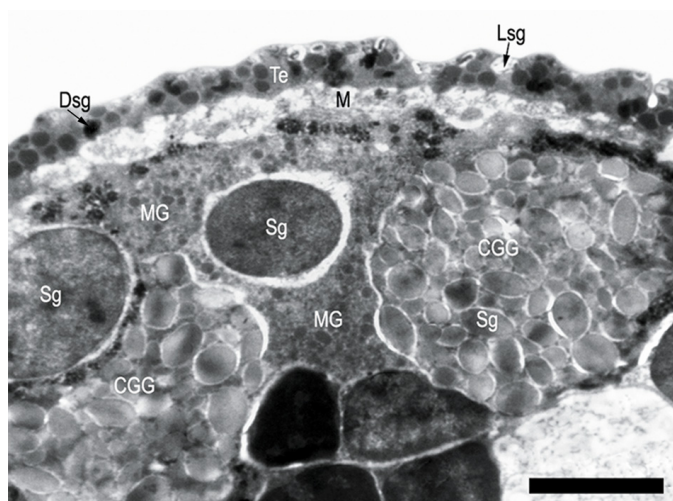
Fig 5–Transmission electron micrograph of the cystogenous glands of a *O. viverrini* cercaria. CGG, cystogenous gland, as semitranslucent electron dense granules; M, muscle; Te, tegument; Dsg, dense spherical granule; Lsg, light spherical granule; MG, mucoid gland; Sg, secretory granule. Scale bar= 200 nm.

smaller than that previously recorded by Adam *et al* (1993). The differences in sizes might be due to difference in the method of sample fixation, preparation of the slides or the program used for measurement. *O. viverrini* cercariae are a type of pleurolophocercous cercariae (Ito *et al*, 1962; Wykoff *et al*, 1965; Harinasuta, 1969) similar to parapleurolophocercous cercariae (Pinto and Melo, 2010; Krailas *et al*, 2011) found in *Bithynia siamensis siamensis* (Chontananarth *et al*, 2013). Parapleurolophocercous cercariae develop into minute intestinal fluke species, such as *Centrocestus caninus*, *Haplorchis taichui* and *Haplorchis pumilio*. They may be designated as either pleurolophocercous or parapleurolophocercous cercariae depending on if the lateral fin-folds were absent or present, respectively. The fin-folds of pleurolophocercous cercariae are composed of ventral and dorsal parts (Wykoff *et al*, 1965; Schell, 1970; Chonta-

nanarth *et al*, 2013). Confirmation of *O. viverrini* cercariae identified morphologically was made using specific *O. viverrini* primers similar to the study of Wongratanacheewin *et al* (2001).

The cercariae were sectioned and stained with H&E. The cystogenous glands of cercariae were not clear but 5 pairs of penetration glands were clearly observed and massed together behind the pharynx as described previously (Wykoff *et al*, 1965). In the present study, the location of the cystogenous glands was confirmed by immunoperoxidase and immunofluorescence staining. The antigen and antibody reactions were seen mostly at the lateral part of the body of the cercariae but the location of the cystogenous glands was still uncertain. It has been shown that the cystogenous glands of *Echinostoma revolutum* cercariae are found packed at the dorsal part along the whole length of the body (Žd'árská and Našincová, 1985; Žd'árská, 1989). The cystogenous glands of *O. viverrini* and *C. shikokuensis* cercariae were previously thought to be located dorsolaterally on both sides of the cercariae (Wykoff *et al*, 1965; Harada and Suguri, 2001).

The antigen and antibody reactions were positive at other locations or other glands. This may be due to: 1) the outer cyst wall not being removed from the cyst wall of metacercariae during antigen preparation, which could have affected pure antigen production. The cyst wall of the metacercariae has been shown to be covered with a double-wall (Vajrasthira *et al*, 1961) with the outer thick wall be-

ing produced from defense mechanisms of fish and the inner thin wall resulting from secretions from the cystogenous glands of the cercariae. The inner wall is composed of cystogenous substances (Žd'árská and Našincová, 1985; Žd'árská, 1989; Harada and Suguri, 2001). The cyst of *O. viverrini* metacercariae was molded 20 minutes after cercariae had penetrated hosts (Donthaisong *et al,* unpublished data). At 3 day post-infection, cysts were formed in the fibrous tissue of fish due to host parasite interactions (Schell, 1970; Donthaisong *et al,* unpublished). 2) The antigen might not have been purified before it was used to immunize the mouse. 3) Secretions from the larvae and other glands were not completely removed from the cyst wall of metacercariae. The other glands in the trematode cercariae were the ventral glands located at the ventral half of the body. The mucoid glands were distributed around the ventral sucker, the dorsal and dorsolateral surface of the body, and located between the cystogenous glands. The penetration glands were found in the anterior half of the body (Žd'árská and Našincová, 1985; Žd'árská, 1989; Galaktionov and Malkova, 1994; Harada and Suguri, 2001). 4) Many types of substances originate from the cystogenous glands are the same as the other glands of the cercariae. The cystogenous substances of trematode cercariae have been shown to be acid mucopolysaccharide, neutral mucopolysaccharide, and keratin protein. The penetration, ventral, and mucoid glands are similar to the cystogenous glands, namely, acid mucopolysaccharide and neutral mucopolysaccharide (Dixon, 1966; Žd'árská, 1979; Žd'árská and Soboleva, 1980; Bakar and Nollen, 1986; Harada and Suguri, 2001). 5) The protein components of the cyst wall were of the same protein types found in *Clonorchis sinensis*:

C. sinensis enolase and *C. sinensis* 14-3-3. These proteins were deposited on the cyst wall of *C. sinensis* metacercariae. Thus, the cyst wall components of *O. viverrini* may be similar to those of *C. sinensis* (Wang *et al,* 2011, 2012). Previous research has found the cyst wall of trematode metacercariae (*F. hepatica*, *Parorchis acanthus*, *Bucephalus haimeanus*, *E. revolutum*, and *C. shikokuensis*) were mucoprotein, protein (cysteine, cystine, and phospholipids), acid mucopolysaccharide, neutral mucopolysaccharide, lipoprotein, glycoprotein, tanned-protein, mucoprotein, lipid, and keratin protein (Dixon and Mercer, 1964; Dixon, 1965; Dixon, 1966; Rees, 1967; Higgins *et al,* 1977; Žd'árská and Našincová, 1985; Harada and Suguri, 2001). However, cyst wall components of *F. hepatica* metacercariae which are encysted on watercress or other aquatic plants (Dow *et al,* 1968), may be different from *O. viverrini* metacercariae which encyst in fish tissue (Schell, 1970).

The immunolocalization of mature metacercariae was strongly presented as positive reactions in the cyst wall and weakly presented in the larval body. At 10 days post-infection, they were weakly presented in the cyst wall and strongly presented in the larval body. The cyst wall antigens of the metacercariae and fish were produced in mice to prepare antibodies which cross reacted with the cysts in fish. The cyst wall components of metacercariae might be different between mature metacercariae and metacercariae at 10 days post-infection because of different mechanisms of infecting fish. The cystogenous substances of mature metacercariae have been used in the process of the inner cyst wall formation (Žd'árská and Našincová, 1985; Žd'árská, 1989; Harada and Suguri, 2001) or they metabolized the substance to produce energy for the

larva, which may influence the loss of the cystogenous substances from the larval body. At 10 days post-infection, positive reactions occurred in the larval body because of excess cystogenous substances remaining in the cystogenous glands of the larva. While collecting the cyst wall we could not wash out the protein contents within the cyst such as some excretion from larval tegument (the granules in tegument) which could be used as antigen to induce antibody resulting in a positive reaction within the tegument of the larva.

Ultrastructural examination showed that the cystogenous glands were located dorsolaterally in the cercariae. This gland was packed with many semitranslucent electron dense oval shaped granules. These is similar to *Cryptocotyle lingua* cercariae which have many oval shaped granules (Matthews and Matthews, 1993). Žd'árská and Našincová (1985) described the cystogenous glands of *E. revolutum* as club shaped and narrow. When the cystogenous glands cells had developed, they contained rod shaped granules (Žd'árská, 1989). These granules were released from the cystogenous glands (Harada and Suguri, 2001), including penetration and mucoid glands (Galaktionov and Malkova, 1994). The granules, also known as secretory bodies or secretory granules, are oval shaped and electron dense (Southgate, 1971; Rees and Day, 1976; Galaktionov and Malkova, 1994). The tegument of first-week *O. viverrini* juveniles and adult stages consisted of many dense and light granules (Apinhasmit *et al*, 1994). It has been suggested that these granules are the source of the outer plasma membrane and the glycocalyx (Hanna, 1980; Apinhasmit *et al*, 1994).

The present study describes the location of *O. viverrini* cercariae cystogenous glands including the shape and size of the cystogenous granules. The location of the cystogenous glands was the same as that shown by immunoperoxidase. The morphology of the cystogenous granules was the same as that reported by Matthews and Matthews (1993). Further investigations should be conducted to identify the components and protein types in cystogenous glands which might suppress the gland function to enable the formation of the cyst wall of *O. viverrini* metacercariae.

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