นิพนธ์ต้นฉบับ • Original Article

รูปร่างลักษณะของอัณฑะของพยาธิใบไม้ตับ *Opisthorchis viverrini* ตัวเต็มวัย

ศิริลักษณ์ วรรณปะโพธิ์[1], สมาน เทศนา[2], ชาญณรงค์ อรัญนาถ[1], พิพัฒน์พงษ์ แคนลา[1], วรรณี ชัยจรูญคณารักษ์[1]
[1]ภาควิชากายวิภาคศาสตร์ [2]ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น 40002

Morphological Features of the Testes of the Adult Liver Fluke, *Opisthorchis viverrini*

Siriluk Wannaprapo[1], Smarn Tesana[2], Channarong Arunyanat[1], Pipatpong Kanla[1], Wunnee Chaijaroonkhanarak[1]
Department of [1]Anatomy, [2]Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand.

หลักการและเหตุผล: พยาธิใบไม้ตับ *Opisthorchis viverrini* พบระบาดในแถบภาคตะวันออกเฉียงเหนือของประเทศไทย รวมทั้ง ลาว กัมพูชา และทางตอนใต้ของเวียดนาม พยาธิใบไม้ตับชนิดนี้เป็นปัจจัยสำคัญของการเกิดโรคเกี่ยวกับระบบท่อทางเดินน้ำดีในคน พยาธิใบไม้ตับเพียงตัวเดียวสามารถผสมพันธุ์เองได้แบบ self-fertilization อวัยวะสืบพันธุ์เพศผู้ประกอบด้วย อัณฑะสองอันมีลักษณะเป็นกลีบอยู่บริเวณท้ายลำตัวใกล้กับรังไข่ ยังไม่มีรายงานการศึกษาระดับจุลกายวิภาคศาสตร์ของอัณฑะของพยาธิใบไม้ตับ *Opisthorchis viverrini*

วัตถุประสงค์: เพื่อศึกษารูปร่างลักษณะทั่วไปของอัณฑะของพยาธิใบไม้ตับ *O. viverrini* ตัวเต็มวัย

รูปแบบการศึกษา: ศึกษาลักษณะทั่วไปของอัณฑะของพยาธิใบไม้ตับ *O. viverrini* ตัวเต็มวัยด้วยกล้องจุลทรรศน์แบบธรรมดา (Light Microscope, LM) และกล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่านและแบบส่องกราด (Transmission and Scanning Electron Microscopes, TEM and SEM)

สถานที่ทำการศึกษา: ภาควิชากายวิภาคศาสตร์ และภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น

วิธีการศึกษา: นำพยาธิใบไม้ตับ *O. viverrini* ระยะ encyst เมตาเซอร์คาเรีย ที่ได้จากปลาที่ติดเชื้อพยาธิ ป้อนเมตาเซอร์คาเรียให้แก่หนูแฮมสเตอร์ (Golden Syrian hamsters) 10 ตัว โดยวิธี gastric intubation หนูแต่ละตัว ได้รับเมตาเซอร์คาเรียจำนวน 50 ตัว เลี้ยงหนูจนครบ 28 วัน จึงฆ่าหนูโดยสลบด้วย ether จากนั้นนำพยาธิออกจากตับและทางเดินน้ำดีเพื่อเข้าสู่กระบวนการเตรียมตัวอย่างสำหรับศึกษาด้วยกล้อง

Background: Infection of *Opisthorchis viverrini* is widely endemic mainly in Northeast of Thailand including Laos, Cambodia and South Vietnam. Its prevalence and pathogenic effects are associated with several hepatobiliary diseases in human. The liver fluke is monoecious, seft-fertilization can be occurred individually. The male reproductive organ usually consists of two deeply lobed testes, located in the posterior region of the body next to the ovary. No description is available on the ultrastructure and development of the testes of the *Opisthorchis viverrini.*

Objectives: This study aims to examine the morphology and ultrastructure of the testes of the adult flukes *O. viverrini.*

Study design: Descriptive study based on morphological observation by using light microscope (LM), transmission electron microscope (TEM) and scanning electron microscope (SEM).

Setting: Department of Anatomy and Department of Parasitology, Faculty of Medicine, Khon Kaen University, Thailand.

Materials and Methods: The encyst metacercaria of *Opisthorchis viverrini* were identified and collected. Ten adult Golden Syrian hamsters were used. Each animal was infected with 50 encyst metacercaria by gastric intubation. The animal were then sacrificed by deep ether anaesthesia, at 28 days post infection. The recovered flukes were routinely processed for investigation of the testes by LM, TEM and SEM.

จุลทรรศน์แบบธรรมดา และกล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่านและแบบส่องกราด

ผลการศึกษา: การศึกษาด้วยกล้องจุลทรรศน์ธรรมดาในตัวอย่าง whole mount ของพยาธิใบไม้ตับ *Opisthorchis viverrini* พบอัณฑะ 2 ชุด อยู่ประมาณ 1/3 ทางด้านท้ายของลำตัวหลังต่อรังไข่ อัณฑะแต่ละชุดมีลักษณะเป็นกลีบ จำนวน 4-5 กลีบ ภายในอัณฑะประกอบด้วยเซลล์สืบพันธุ์ระยะต่างๆ ได้แก่ spermatogonia อยู่ชิดขอบของ testicular wall บริเวณลึกเข้ามาพบ spermatocytes และ spermatids โดย nucleus ของ early spermatids มีลักษณะยาวพบ lamellar chromatin และ striated rootlets จำนวน 2 ชุด การศึกษาด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด พบว่าตัวอสุจิมีรูปร่างลักษณะเรียวยาว

สรุป: การศึกษาครั้งนี้เป็นการรายงานครั้งแรกเกี่ยวกับรูปร่างลักษณะทั่วไปของอัณฑะของพยาธิใบไม้ตับ *Opisthorchis viverrini* ระยะตัวเต็มวัยด้วยกล้องจุลทรรศน์ธรรมดาและกล้องจุลทรรศน์อิเล็กตรอน พบว่าภายในอัณฑะประกอบไปด้วยเซลล์สืบพันธุ์ระยะต่างๆ ได้แก่ spermatogonia spermatocytes และ spermatids รวมกลุ่มกันเป็น rosette ส่วนตัวอสุจิมีรูปร่างเป็นเส้นเรียวยาว

Results: In the whole mount specimens, the pair testes of the adult *O. viverrini* were clearly defined at the posterior 1/3 of the worm body posterior to the ovary. They were multilobate, 4-5 lobes each and located one behind the other. The testes showed numerous germ cells at various stages of development. The spermatogonia were closest to the testicular wall. In the deeper regions, cluster of numerous spermatocytes and spermatids were observed. The early spermatid exhibited an elongated nucleus with exhibiting lamellar chromatin alongside the two sets of striated rootlets. The scanning electron microscopic studied revealed that the spermatozoa were long filiform-shaped.

Conclusions: This is the first report of the morphology of the testes of the adult flukes, *Opisthorchis viverrini* studied by using light and electron microscopes. The testes contained different stages of developing spermatogenic cells. Their three dimentional morphology were thread-like with no easily discernible heads and tails.

Keywords: *Opisthorchis viverrini*, spermatogenic cells, testes, ultrastructure

ศรีนครินทร์เวชสาร 2551; 23(4): 346-52 • Srinagarind Med J 2008; 23(4): 346-52

Introduction

The human liver fluke, *Opisthorchis viverrini* is widely and extremely distributed in E-San or Northeast of Thailand including several countries in Southeast Asia such as Laos PDR, Cambodia and South Vietnam.[1] Its prevalence and pathogenic effects are associated with the fatal hepatic cancer of the bile duct epithelia, known as cholangiocarcinoma.[2] The liver fluke is monoecious. The adult flukes reside in biliary passages of the liver in which sexual reproduction occur. Sex organs are the two lobed testes and the multilobated ovary which situated rostal to both testes. The ultrastructures of the testes have not been discovered. The present study could provide more detailed informations on some aspects of biology of the *Opisthorchis viverrini*. We aim to examine the morphology and ultrastructures of the adult testes of the liver fluke, *Opisthorchis viverrini* recovered from biliary system of the Golden Syrian hamsters, by light and electron microscopes.

Materials and Methods

Opisthorchis viverrini metacercariae were obtained from naturally infected cyprinoid fish in endemic area in the Northeastern region of Thailand, Khon Kaen province. The whole fish were minced and then treated with a solution containing pepsin and 0.25% hydrochloric acid. The digested fish muscle were then passed through three graded sieves, mesh size of 850, 300 and 250 respectively. The active encysted metacercaria of *Opisthorchis viverrini* were identified and collected under a stereomicroscope. Ten adult Golden Syrian hamsters from animal center Khon Kaen University were used. Each animal was infected with 50 encysted metacercariae by gastric intubation to obtain the adult flukes. All animals were fed *ad libitum* for 4 weeks after infection, then sacrificed by deep ether anaesthesia. The living adult flukes were removed from the hamsters's biliary tract. The recovered flukes were fixed in 10% neutral

formalin. Whole mount specimens were stained with Semichon's acid carmine, cleared in methyl salicylate, mounted in Canada balsam and finally viewed with a light microscope and measured microscopically. Another ten parasites were fixed in modified Karnovsky's fixative for 2 hours and post-fixed in 1% Osmium tetroxide. After dehydration through a graded series of ethanol, the specimens were embedded in araldite. Semithin sections 1 um thick were cut, then stained with 1% toluidine blue and 1% basic fuchsin and examined with a light microscope. The ultrathin sections with 70 nm were cut using an LKB ultramicrotome and picked up on copper grids (size 150 mesh). They were stained sequentially with uranyl acetate followed by lead citrate, examined and photographed under a HITACHI H 600 transmission electron microscope at accelerating voltage 75 Kv. For scanning electron microscopic study, after dehydration the specimens were critical point dried and mounted on the metal stub and coated with gold. The specimens were then viewed and photographed using a HITACHI-S200N scanning electron microscope at the accelerating voltage of 10 Kv. This study was approved by the Animal Ethic committee, Faculty of Medicine, KKU (Ref No: AE009/50).

Results

We found that in the whole mount specimens, the average body size of the adult *O. viverrini* was 0.56 mm in width and 1.26 mm in length. The pair testes were clearly defined at the posterior 1/3 of the worm body posterior to the ovary. They were multilobate, 4-5 lobes each and located one behind the other. They appeared on both sides of the S - shaped excretory bladder and were placed between the intestinal ceca (Figure 1). The average areas of the anterior and the posterior testes were 0.02 mm^2 and 0.017 mm^2, respectively. In semithin sections the testes showed numerous spermatogenic cells at various stages of development. The spermatogonia were irregular in shape and appeared as 1-3 cell layers closest to the testicular wall (Figure 2). Observation by using TEM, they possessed prominent large, centrally placed spherical nuclei with disperse heterochromatin and 1-2 nucleoli. The relatively wide perinuclear spaces and nuclear pores were evidenced. The cytoplasm contained numerous mitochondria, ribosomes, rough endoplasmic reticulum and vacuoles. A nucleolus-like body was noticed in some spermatogonia (Figure 3). In the deeper region, clusters of numerous spermatocytes were observed. They were larger than the spermatogonia with spherical nuclei and various stages of condensation. Synaptonemal complex was observed in the nucleus of the spermatocyte (Figure 4). This structure was formed by two lateral electron dense lines sandwiched together with a central clear zone. The perinuclear spaces, nuclear pores and nucleolus-like bodies were also evidenced. Numerous rosettes formed from aggregation of early spermatids joined by a central cytophore were observed (Figure 2, 5). Periphery rosettes of spermatids were relatively infrequent (Figure 2). The spermatids were the smallest cell during spermatogenesis. Several small clumps of chromatin were distributed in the nucleus of early spermatids (Figure 5). The differentiation zone of the spermatid was marked by arched membranes (Figure 6, 7). This area contained elongated nucleus with exhibiting lamellar chromatin alongside the two sets of striated rootlets (Figure 6) associated with basal bodies and a pair of axonemes. The axoneme exhibited a 9+1 pattern of microtubules. In transverse sections, numerous anterior extremities of the spermatozoa with single axoneme were observed. Whereas the posterior region contained a round nucleus with condensed chromatin (Figure 8). Observations by using scanning electron microscope revealed that the spermatozoa were long filiform shaped (Figure 9).

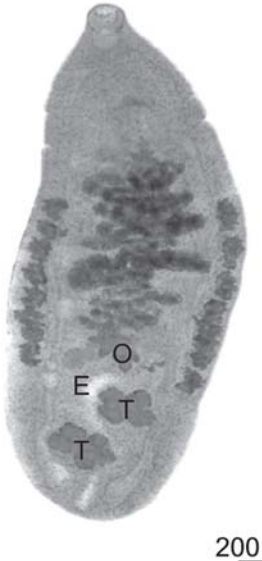

Figure 1 Whole mount specimen of the adult *O. viverrini.* T, testis; E, excretory bladder; O, ovary.

Transmission electron micrographs figure 3-8.

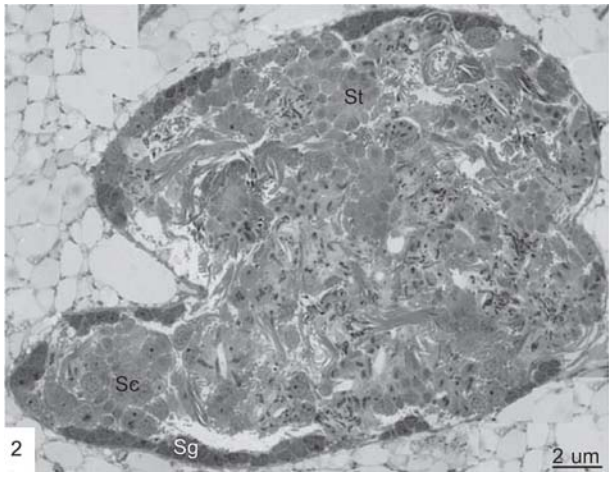

Figure 2 Light micrograph of lobation of the testes. Numerous spermatogenic cells in various stages of development. Sg, spermatogonia; Sc, spermatocytes; St, spermatids.

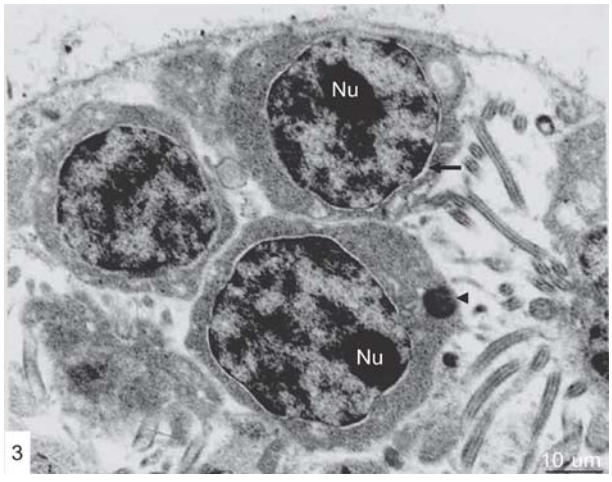

Figure 3 Peripheral region of the testicular tube, showing the spermatogonia with prominent heterochromatic nuclei and wide perinucleolar space (black arrow). Nucleolus (Nu), nucleolus-like body (arrowhead).

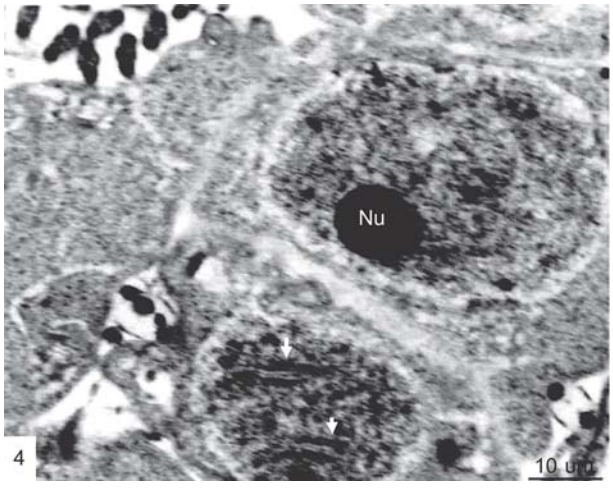

Figure 4 Spermatocytes showing synaptonemal complex (white arrows); Nu, nucleolus.

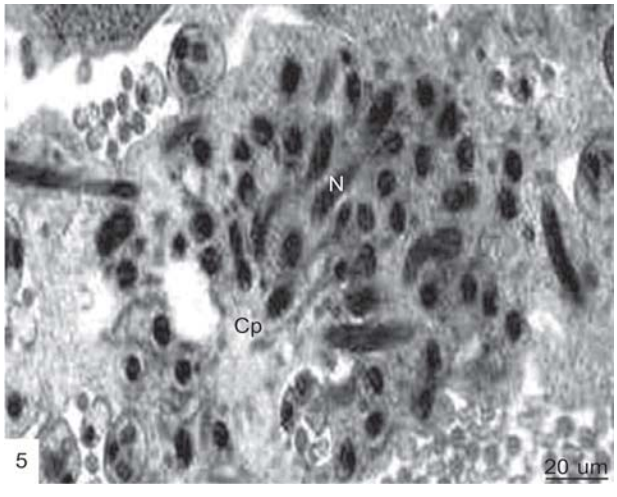

Figure 5 Rosette of spermatids connected by a central cytoplasmic mass, the cytophore (Cp). Several longitudinal sections of spermatids with elongated nuclei (N) were observed.

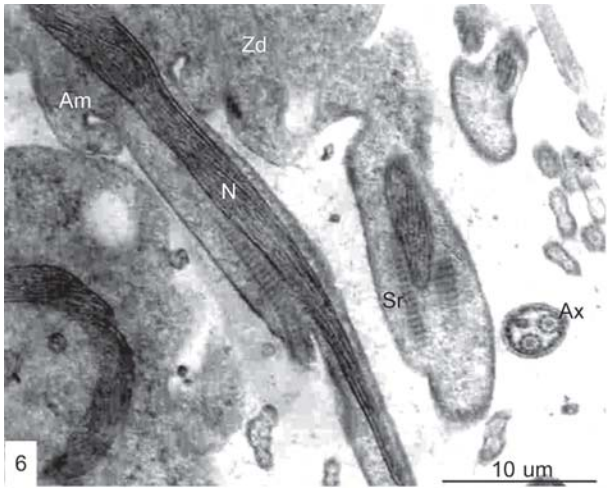

Figure 6 Zone of differentiation (Zd) of spermatids showing the arch membrane (Am), elongated nucleus (N) with lamellar chromatin, striated rootles (Sr). Ax, a cross section of 2 axonemes (Ax) with peripheral microtubules.

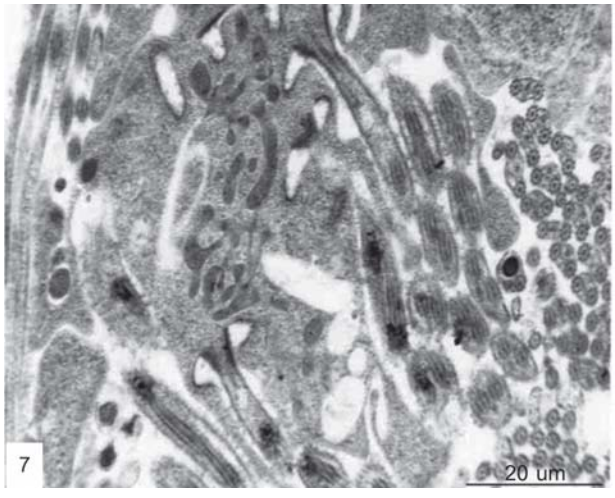

Figure 7 Severals long and cross sections through the spermatids.

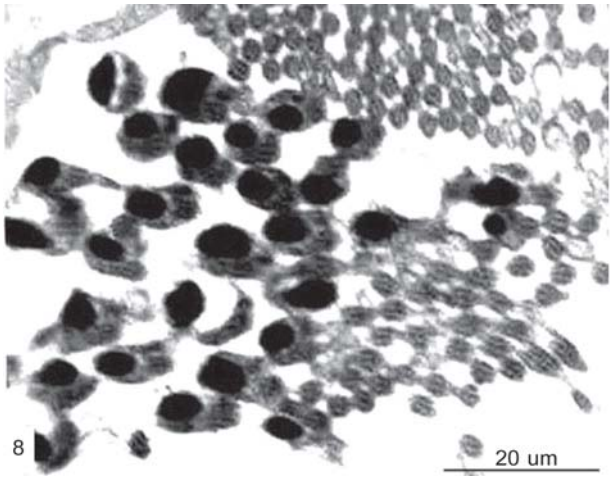

Figure 8 Transverse and cross sections of spermatozoa showing the condensed nuclei.

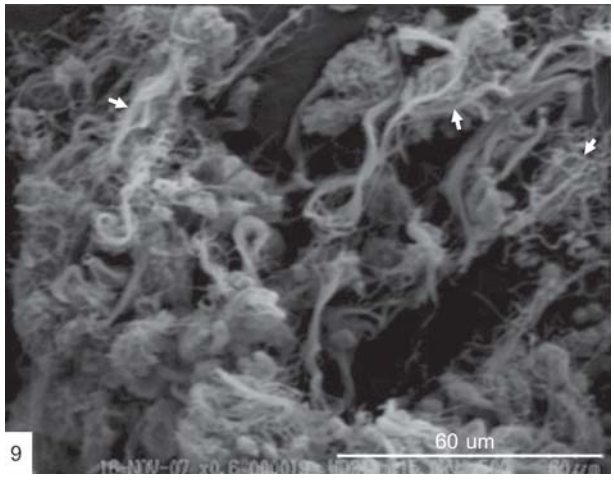

Figure 9 Scanning electron micrograph showing the long filifrom shaped spermatozoa (arrows).

Discussions

In this study we examined the morphological features of testes of the adult *Opisthorchis viverrini*. The testes were divided into 4-5 lobes. Both testes were about the same size. Nithikathkul *et al.*, [3] reported that the testes of the *O. viverrini* were present in the first week and were lobulated after one and a half weeks. Therefore, the testes of the juvenile *O. viverrini* showed lobes as in the adult at about 10 days after infected into the Golden Syrian hamsters. Whereas on development of *C. sinensis* in the guinea pigs, the testes became lobulated as in the adult on day 7.[4] Observations on the testes of the adult *O. viverrini* showed the spermatogonia with prominent nuclei occupied most of the cytoplasm similar to the study of Baptista-Farias *et al.*[5] The presence of synaptonemal complex between homologous chromosomes within the nuclei in a number of cells indicated that they

were primary spermatocytes[6] which in the zygotene stage of prophase of meiosis I. This pairing or "synapsis" of homologus chromosomes were early described in primary spermatocytes of the invertebrate crayfish[7] including the trematode Digenea, Dicrocoeliidae[6] and Haploporidae[5] and in spermatocytes of pigeon, cat and man.[8] Baptista-Farias et al.[9] recognized the presentation of synaptonemal complex to differentiate the primary and secondary spermatocytes. Jeong et al.[10] used the 8-cell and 16-cell groups to identify the primary and secondary spermatocytes on their light microscopic observations of the extracted specimens of the testes of *C. sinensis*. In the present study, there were no prominent features to distinguish these two stages of spermatogenic cells. Likewise, the nucleolus-like bodies in the cytoplasm of a number of cells were similar to the first report in *C. vitta*.[6] The appearance of the cytophore in the spermatogenic cells in *O. viverrini* were also identified in others trematodes[6,9,10] and some cestodes.[7] This structure had not been determined in other animals during spermatogenesis.[7] Differentiation zone was observed in early spermatogenesis of the *O. viverrini*. The elongated nucleus with lamellar chromatin was markedly contrast to the nucleus in other stages of development. The elongate spermatozoa contain one flagellum formed from corporation of pair axonemes were previously observed in others digenean trematodes such as Brachylaimidae,[11] Fasciolidae,[12] Dicrocoeliidae,[13] Haploporidae[5] and Deropristidae.[14] The presence of 2 axonemes with cortical microtubules was classified as type 1 spermatozoon previously described by Justine[15] The long filiform-shaped spermatozoon of *O. viverrini* as seen in SEM was similar to most observations on spermiogenesis and ultrastructures of the sperm.[11, 15-17]

Acknowledgements

This work was financially supported by the Graduate School and Invitation Research Grant from Faculty of Medicine, Khon Kaen University. We thank Emeritus Professor Dr. Somboon Srungboonmee for assistance with English language preparation of the manuscript.

References

1. Harinasuta T, Riganti M, Bunnag D. *Opisthorchis viverrini* infection: pathogenesis and clinical features. Arzneimitte forschung 1984; 34:1167-9.
2. Sripa B, Kanla P, Sinawat P, Haswell-Elkins MR. Opisthorchiasis-associated biliary stones: Light and Scanning electron microscopic study. World J Gastroenterol 2004; 10: 3318-21.
3. Nithikathkul C, Tesana S, Sithithawon P, Balakanich S. Early stage biliary and intrahepatic migration of *Opisthorchis viverrini* in the golden hamster. J helminthol 2007; 81:39-41.
4. Komiya Y. *Clonorchis* and clonorchiasis. Advances Parasitol 1996; 4:53-106.
5. Baptista-Farias MDF, Kohn A, Cohen SC. Ultrastructure of spermiogenesis and sperm development in *Saccocoelioides godoyi* Kohn & Froes, 1986 (Digenea, Haploporidae). Mem Inst Oswaldo Cruz, Rio de Janeiro 2001; 96:61-70.
6. Robinson RD, Halton DW. Fine structural observations on spermatogenesis in *Corrigia vitta* (Trematoda: Dicrocoeliidae). Z Parasitenkd 1982; 68:53-72.
7. Moses MJ, Chromosomal structures in Crayfish spermatocytes. J Biophysic Biochem Cytol 1956; 4:633-38.
8. Fawcett DW. The Fine Structure of chromosomes in the meiotic prophase of vertebrate spermatocytes. Journal Biophysic Biochem Cytol 1956; 2:403-11.
9. Baptista-Farias MDF, Kohn A, Cohen SC. Spermatogenesis and Spermiogenesis in Microcotyle sp. (Microcotylidae, Monogenea). J Parasitology 1999; 85: 832-8.
10. Jeong KH, Rim HJ, Yang HY, Kim WK, Kim CW. A morphological study on spermatogenesis in the liver fluke, *Clonorchis sinensis*. Korean J Parasitol 1976; 14: 123-32.
11. Ndiaye PI, Feliu C, Marchand B. Spermiogenesis and sperm ultrastructure of *Scaphiostomum palaearcticum* Mas-Coma, Esteban et Valero, 1986 (Trematode, Digenea, Brachylaimidae). Acta Parasitol 2002; 47: 259-71.
12. Ndiaye PI, Miquel J, Bâ CT, Marchand B. Spermiogenesis and ultrastructure of the spermatozoon of the liver fluke *Fasciola gigantica* Cobbold, 1856 (Digenea: Fasciolidae), a parasite of cattle in Senegal. J Parasitol 2004; 90:30-40.
13. Agostini S, Miquel J, Ndiaye PI, Marchand B. *Dicrocoelium hospes* Looss, 1907 (Digenea, Dicrocoeliidae): Spermiogenesis, mature spermatozoon and ultrastructural comparative study. Parasitol Res 2005; 1:38-48.
14. Foata J, Quilichini Y, Marchand B. Spermiogenesis and sperm ultrastructure of *Deropristis Inflata* Molin, 1859 (Digenea, Deropristidae), a parasite of *Anguilla anguilla*. Parasitol Res 2007; 101:843-52.

15. Justine JL. Spermatozoal ultrastructure and phylogeny of the parasitic Plathehilminthes. In: BGM Jamieson, J Ausio, JL Justine, eds, Advances in Spermatozoal Phylogeny and Taxonomy, Paris: Mem Mus Natn Hist Nat, 1995:55-86.
16. Miquel J, Nourrission C, Marchand B. Ultrastructure of spermiogenesis and the spermatozoon of *Opecoeloides furcatus* (Trematoda, Digenea, Opecoelidae), a parasite of *Mullus barbatus* (Pisces, Teleostai). Parasitol Res 2000; 86:301-10.
17. Ndiaye PI. Spermiogenesis and sperm ultrastructure of the liver fluke *Fasciola hepatica L.*, 1758 (digenea, Fasciolidae): transmission and scanning electron microscopy, and tubulin immunocytochemistry. Acta Parasitol 2003; 48:182-94.