RESEARCH NOTE

POTASSIUM PERMANGANATE STAINING FOR DIFFERENTIATION THE SURFACE MORPHOLOGY OF *OPISTHORCHIS VIVERRINI*, *HAPLORCHIS TAICHUI* AND *PHANEROPSOLUS BONNEI* EGGS

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Abstract. Potassium permanganate staining method was developed for differentiation *Opisthorchis viverrini*, *Haplorchis taichui* and *Phaneropsolus bonnei* eggs. The surfaces of *O. viverrini*, *H. taichui* and *P. bonnei* eggs stained permanently and temporarily were similar in appearance even the staining procedures were varied both in concentration and time. Determined under light microscope set at 400x, all of these eggs were oval-shaped, operculated at one pole and indistinct small knob at posterior end. *O. viverrini* eggs showed the distinct musk-melon-like prominent ridges on the surface. *Haplorchis taichui* eggs had a light striae pattern while *P. bonnei* eggs had a smooth egg shell. Length of these trematode eggs were significant different (χ^2 test, $p < 0.05$). Mean ± SD of *O. viverrini*, *H. taichui* and *P. bonnei* eggs were 26.34 ± 1.65 μm, 29.03 ± 1.48 μm and 23.00 ± 1.49 μm, respectively. Regarding of their width, the mean ± SD of *O. viverrini*, *H. taichui* and *P. bonnei* eggs were 15.54 ± 0.69 μm, 14.94 ± 0.91 μm and 12.25 ±1.02 μm, respectively. The means of width of *O. viverrini* and *H. taichui* eggs were not significantly different (χ^2 test, $p > 0.05$), however, they were significantly different from those of *P. bonnei* (χ^2 test, $p < 0.05$). Temporary staining using 1% w/v concentration and only 1 minute of time is useful in the mass fecal examination survey for the prevalence and intensity of truly *Opisthorchis* infection.

Opisthorchis viverrini infection is the most important trematode infection in Thailand with the serious manifestations of obstructive jaundice and cholangiocarcinoma (Preuksaraj, 1984). However, there are many species of minute intestinal flukes found in Thai people (Manning *et al*, 1971; Kaewkes *et al*, 1991a; Radomyos *et al*, 1994). These minute intestinal flukes are of lesser clinical importance than *Opisthorchis* infection, nevertheless, the prevalence of these minute flukes from human cases in northern Thailand has been reported frequently (Radomyos *et al*, 1998). Diagnosis of *O. viverrini* infection is routinely based on the finding of its eggs in human feces. However, the eggs of *O. viverrini* and other minute intestinal flukes of Heterophyidae and Lecithodendriidae are practically indistinguishable one from another by routine stool examination using light microscope (Ditrich *et al*, 1990; Tesana *et al*, 1991). The surface ultrastructure of these eggs under scanning electron microscope have previously been shown to provide familial differentiation of most of these flukes (Ditrich *et al*, 1990; Kaewkes *et al*, 1991b; Tesana *et al*, 1991). The musk-melon pattern of opisthorchid eggs, the flat thread-like ridge pattern of heterophyid eggs and the smooth surface pattern of lecithodendriid eggs have been noted (Tesana *et al*, 1991), however, this is not useful as a routine technic in the diagnostic laboratory (Ditrich *et al*, 1990). This pattern of surface morphology of *O. viverrini* eggs seen under scanning electron microscope has also been claimed to be observed under a good light microscope with very high magnification (Suzuki 1983; Ditrich *et al*, 1992). This study, set out to differentiate between *O. viverrini* and other minute intestinal flukes eggs by using potassium permanganate staining as determined by low magnification light microscope for routine stool examination.

Opisthorchis viverrini, *Haplorchis taichui* and *Phaneropsolus bonnei* were recruited in this study as the representatives of flukes in families Opisthorchiidae, Haplorchiidae and Lecithodendriidae, respectively. The adult worms of *O. viverrini* were collected from the autopsy cases at Maharaj Nakhon Chiang Mai Hospital, Chiang Mai University. The adult worms of *H. taichui* were collected from mice 7 days after experimental infection with the metacercarial stage and *P. bonnei* were kindly provided

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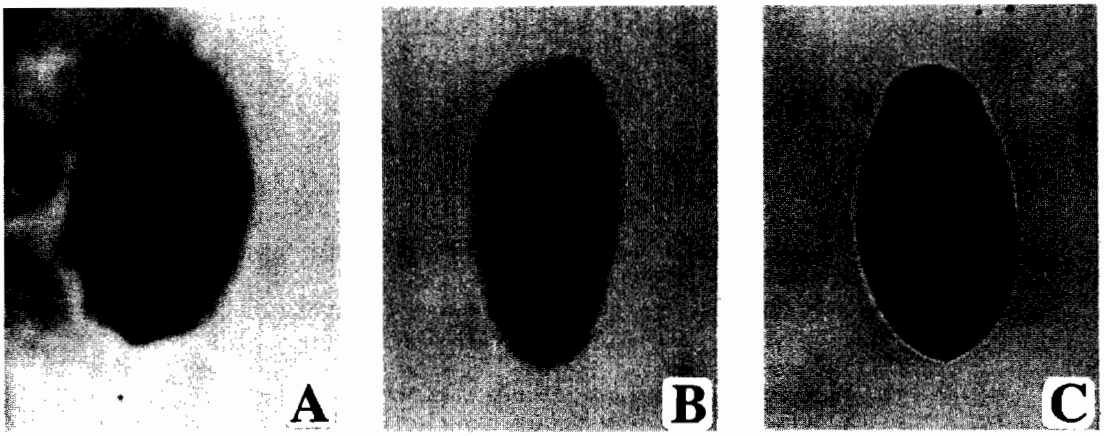

Fig 1–Light micrographs of the egg shell of *Opisthorchis viverrini* (A), *Haplorchis taichui* (B) and *Phaneropsolus bonnei* (C) eggs stained with potassium permanganate and examined under magnification of 400x.

by Professor Radomyos, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. All adult worm specimens were collected in 10% formalin. The upper one third uteri were dissected under a stereo dissecting microscope and then torn to obtain mature eggs. These eggs were then collected in small vials of 10% formalin.

Staining procedure: For permanent stain, one drop of preserved eggs was smeared over the clean glass microscopic slide (diameter 1 cm) using a sterile Pasteur pipette. The films were allowed to remain dry at room temperature for 2 hours. Staining of these films was done by potassium permanganate solution over a concentration range of 1,2,5,10,20,30 and 50 % (w/v), each of which was also varied for 1, 2 and 5 minutes. The stained slides were dehydrated in absolute ethanol and mounted by rapid mounting media for microscopy (Entellan®: Merck) with a 22-mm square coverslip. All permanent slides were examined under light microscope at objective lens 40x and eye piece lens 10x. The result was then recorded. For the temporary stain, one drop of the preserved eggs was smeared on a clean glass microscope slide (diameter 1 cm) then applied with one drop of potassium permanganate solution varied at 1, 2, 5, 10, 20, 30 and 50 % (w/v) over the smears and immediately covered with a 22-mm square coverslip. The temporary slides were then examined by light microscope as for the permanent slides.

Egg size assessment: Measurements of the length and width of *O. viverrini*, *H. taichui* and *P. bonnei* eggs were also made. Fifty eggs of each species were used to assess in size. A light microscope set at 400x magnification and equipped with a calibrated ocular micrometer was used to make egg size assessment. The length of each egg was measured from the tip of operculum to the end of knop while the width of each egg was measured at the maximum width.

Potassium permanganate staining determined under light microscope of magnification set at 400x: The surfaces of *O. viverrini*, *H. taichui* and *P. bonnei* eggs stained permanently and temporarily with potassium permanganate were similar in appearance even the staining procedures were varied both in concentration and time. Determined under light microscope set at 400x, these stained egg surface of *O. viverrini*, *H. taichui* and *P. bonnei* are shown in Figs 1A, 1B and 1C, respectively. All of these eggs were oval-shaped, operculated at one pole and indistinct small knob at posterior end. Even though the eggs of these three species are very similar in shape and size, the differentiation of egg shell structure of these trematodes was still detectable. *Opisthorchis viverrini* eggs showed the distinct muskmelon-like prominent ridges on the surface (Fig 1A). *Haplorchis taichui* eggs had a light striae pattern (Fig 1B) while *P. bonnei* eggs had a smooth egg shell (Fig 1C).

Egg size assessment: Comparison of the size measurement of the *O. viverrini*, *H. taichui* and *P. bonnei* eggs in length and width is shown in Table 1 and Fig 2. Regarding their length, the mean ± SD of *O. viverrini*, *H. taichui* and *P. bonnei* eggs were 26.34 ± 1.65 μm, 29.03 ± 1.48 μm and 23.00 ± 1.49 μm, respectively (χ^2 test, $p < 0.05$). Regarding of their width, the mean ± SD of *O. viverrini*, *H. taichui* and *P. bonnei* eggs were 15.54 ± 0.69 μm, 14.94 ±

Table 1
Comparative measurement of eggs of *Opisthorchis viverrini, Haplorchis taichui* and *Phaneropsolus bonnei* eggs.*

Characters of the egg	*O. viverrini*	*H. taichui*	*P. bonnei*
Length			
Mean	26.34	29.03	23.00
Standard deviation	1.65	1.48	1.49
Minimum	23.31	24.61	20.72
Maximum	31.08	31.08	25.90
Width			
Mean	15.54	14.94	12.25
Standard deviation	0.69	0.91	1.02
Minimum	12.95	12.95	10.36
Maximum	16.84	16.84	12.95

* Measurements are in micrometer. Fifty eggs of each species were measured for the egg size assessment.

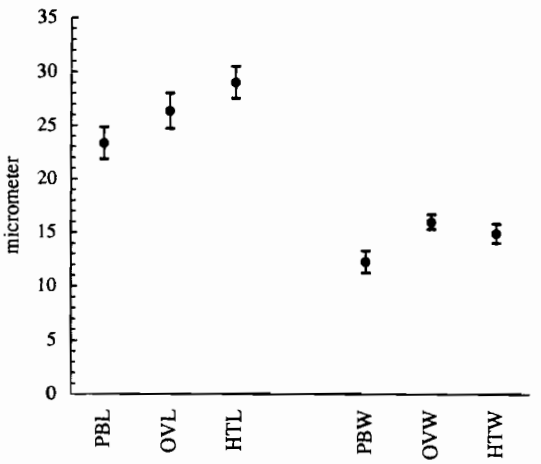

Fig 2–Comparison of the average (± standard deviation) of length and width of *Opisthorchis viverrini*, *Haplorchis taichui* and *Phaneropsolus bonnei* eggs. PBL = *P. bonnei* length, OVL = *O. viverrini* length, HTL = *H. taichui* length, PBW = *P. bonnei* width, OVW = *O. viverrini* width, HTW = *H. taichui* width)

0.91 μm and 12.25 ± 1.02 μm, respectively. The means of width of *O. viverrini* and *H. taichui* eggs were not significantly different (χ^2 test, $p > 0.05$), however, they were significantly different from those of *P. bonnei* (χ^2 test, $p < 0.05$).

Some comparative studies on the surface ultrastructure of opisthorchiid and heterophyid eggs have been reported in both light microscope and scanning electron microscope (Manning *et al*, 1970b; Ditrich *et al*, 1990, 1992; Kaewkes *et al*, 1991b; Tesana *et al*, 1991). Even though the surface structures of eggs examined under scanning electron microscope appeared to be a suitable morphological feature for distinguishing some groups of small flukes (Ditrich *et al*, 1992), it is, however, not suitable for the mass fecal examination in the field (Ditrich *et al*, 1990).

The musk-melon-like ridge on *O. viverrini* eggs stained with potassium permanganate and examined under light microscope (magnification 400x) was clearly presented on the egg surface and accordance with those studies previously described by using scanning electron microscope (Ditrich *et al*, 1990, 1992; Kaewkes *et al*, 1991b; Tesana *et al*, 1991). This unique characteristic of the musk-melon-like ridge was the obvious feature examined under light microscope with the immersion oil objective studied by Ditrich *et al* (1992).

Potassium permanganate stained *H. taichui* eggs revealed the light striae pattern on light microscope at 400x magnification. It may be the same pattern of thread-like structure as found under scanning electron microscope (Tesana *et al*, 1991).

Regarding the eggs of *P. bonnei*, this study revealed that the eggs had no musk-melon or thin striae surface morphological pattern on the egg shell which was correlated with its smooth surface under scanning electron microscope study previously described (Tesana *et al*, 1991). The detailed descriptions with respect to the knob and shoulder of eggs has been reported and revealed that *P. bonnei* eggs had a small protrusion (knob) and the shoulder were not so prominent as occurred in those of *O. viverrini* and *H. taichui* (Tesana *et al*, 1991). In addition, the eggs of lecithodendriid trematodes have been re-

ported to be different from those of *O. viverrini* by the presence of an iodophilic body, a large mass at the posterior end of miracidium that stains brown in 0.2% iodine solution and the curved miracidium of *P. bonnei* (Kaewkes *et al*, 1991b). Stained with iodine and examined under light microscope, the eggs of *P. bonnei* showed tan or brownish color, typical oval shape, moderately thin shell and lack of a distinct shoulder at operculum (Manning *et al*, 1970b). They may have a thin albuminous coat adhering to the egg shell and most do not appear fully developed, but have a vacuolated appearance (Manning *et al*, 1970a). However, all of these descriptions are not easily differentiated by even the expert microscopist.

With regard to the egg size assessment of *O. viverrini, H. taichui* and *P. bonnei* eggs, this study demonstrated that the ranges of their length and width overlapped (Fig 2). In this regard, it was difficult to practically determine the eggs of *O. viverrini* and minute intestinal flukes by size for fecal examination under a light microscope (Ditrich *et al*, 1990; Tesana *et al*, 1991).

Potassium permanganate staining method appeared to be suitable for distinguishing eggs of *O. viverrini* from those of *H. taichui* and *P. bonnei* even though variability as well as similarity in size and shapes of these eggs are still present. Moreover, this staining method made more apparent in egg shell structure than those of the fecal direct smear when it was applied to detect some eggs of nematodes appeared in fecal examination, *ie Ascaris, Trichuris* and hookworm. It was, however, not appropriate for differentiation the intestinal protozoa because the nucleus of these protozoa could not be detected. Since the similar egg shell appearance were obtained even the concentration (1,2,5,10,20,30 and 50% of w/v) and time (1,2 and 5 minutes) of potassium permanganate staining were varied, the lowest concentration (1% w/v) and time (1 minute) were selected as the standard for examining these eggs in this study. Temporary staining will be useful particularly in the mass fecal examination survey for the prevalence and intensity of truly *Opisthorchis* infection.

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