METHYLENE BLUE STAINING METHOD FOR IDENTIFICATION OF *OPISTHORCHIS VIVERRINI* EGG

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Abstract. Methylene blue staining method was used to distinguish *O. viverrini* eggs from *Haplorchis taichui* and *Prosthodendrium molenkampi* eggs. All eggs were obtained from dissected adult worms, fixed in 10% formalin, and stained with methylene blue prior to light microscopy observation. The distinct musk-melon-like texture of the *O. viverini* eggshell surface and the thread-like texture of *H. taichui* eggshell surface were recognized, while *P. molenkampi* eggs showed a smooth eggshell. We also evaluated the sensitivity and specificity of the method by training investigators to differentiate surface textures. After training, the investigators were randomly tested with 10 slides containing fluke eggs. The sensitivity and specificity were 95% and 95%, respectively.

INTRODUCTION

Opisthorchis viverrini is the most common liver fluke in Thailand. Moderate and heavy *O. viverrini* infections are related to obstructive jaundice and cholangiocarcinoma, which cause much suffering and death (Preuksaraj, 1984). Early diagnosis is helpful to prevent severe manifestations. Laboratory diagnosis of the infection is based mainly on stool examination. Since minute intestinal fluke (MIF) eggs are similar in shape and size to *O. viverrini* eggs (Radomyos *et al*, 1984), it is difficult to distinguish *O. viverrini* eggs from MIF eggs. Due to these similarities, the prevalence and intensity of opisthorchiasis can be incorrect and the effectiveness of strategies to minimize *O. viverrini* infection can be difficult to determine.

Although the scanning electron microscope previously demonstrated the detailed surface of these fluke eggs (Ditrich *et al*, 1990), such a method is not suitable for use in the field or routine laboratory. Based on the scanning electron micrscopic evidence, in this study the differences of eggshell surfaces of *Opisthorchis viverrini, Haplorchis taichui* and *Prosthodendrium molenkampi* eggs were compared with a light microscope using the methylene blue staining. The sensitivity and specificity of the method were also evaluated.

MATERIALS AND METHODS

Three worms used in this study were *O. viverrini, H. taichui,* and *P. molenkampi*. Adults were collected from fecal specimens and preserved in 10% formalin at the Department of Parasitology, Faculty of Medical Technology, Mahidol University. The preserved worms were dissected under a stereomicroscope to obtain mature eggs. All eggs were fixed in 10% formalin until used. The stock staining solution (0.16% w/v of methylene blue) consisted of methylene blue 0.016g, azure B 0.1g, anhydrous disodium hydrogen phosphate 1.0g, and monobasic anhydrous potassium phosphate 1.25g in 100 ml of distilled water.

To assess the optimal concentration of methylene blue for a temporary stain, the stock solution was diluted with water to 0.032, 0.016, 0.0016, and 0.0008% (w/v) and *O. viverrini* eggs were stained with various concentrations of the methylene blue solution. We selected the concentration in which the surface of *O. viverrini* eggshells displayed the highest resolution, for use as a working solution for staining of *O. viverrini*, *H. taichui,* and *P. molenkampi* eggshells.

To evaluate the sensitivity, specificity, and accuracy of the method, investigators were trained by demonstrating the differences on the eggshell surfaces of these flukes. Ten stained eggs per species, *O. viverrini, H. taichui,* and *P. molenkampi*, were used as unknown samples for the investigators to examine the eggshell surfaces using a light microscope.

RESULTS

In a direct smear preparation under a light microscope, the eggs of *O. viverrini, H. taichui,* and *P. molenkampi* were oval-shaped, and operculated. *O. viverrini* eggs showed more prominent shoulders and rough skins on the outer surfaces of eggshells than the others. But these differences were not practical to distinguish by species under a light microscope except by experienced technicians.

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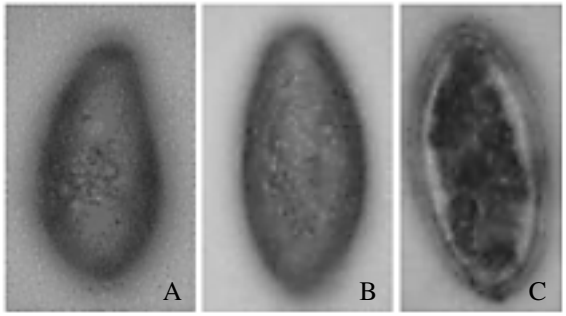

Fig 1- The appearance on surfaces of *Opisthorchis viverrini*(A), *Haplorchis taichui* (B), and *Prosthodendrium molenkampi* (C) eggshells stained with methylene blue under a light microscope at the high magnification of 1,000x

Table 1
Performance of methylene blue staining method for detection of *Opisthorchis viverrini, Haplorchis taichui* and *Prosthodendrium molenkampi* eggs.

Methylene blue staining	*O. viverrini* eggs	*H. taichui* eggs	*P. molenkampi* eggs
False positive (%)	5.0 (0 to 10)	3.8 (0 to 10)	8.8 (5 to 15)
False negative (%)	5.0 (0 to 20)	22.5 (20 to 30)	7.5 (10 to 20)
Accuracy (%)	95.0 (90 to 100)	90.0 (86.7 to 93.3)	91.7 (83.3 to 96.7)
Sensitivity (%)	95.0 (80 to 100)	77.5 (70 to 80)	92.5 (80 to 100)
Specificity (%)	95.0 (90 to 100)	96.3 (90 to 100)	91.3 (85 to 95)

For a temporary stain with methylene blue, the eggshell surfaces of *O. viverrini* were examined under a light microscope. Initial observations indicated that detailed surfaces of *O. viverrini* eggshells could be obviously seen when stained with the methylene blue (0.032% w/v) and visualized at a total magnification of 1,000x (Fig 1). Therefore, we selected this procedure to use as a standard method for staining eggs of *O. viverrini, H. taichui,* and *P. molenkampi*.

The methylene blue stained eggs of *O. viverrini* showed prominent muskmelon-like ridges on the surface. In contrast, *H. taichui* eggs had flat, thread like curly ridges and *P. molenkampi* eggs had smooth surfaces. These differences were shown in Fig 1.

Performance of methylene blue staining for detection of *O. viverrini, H. taichui,* and *P. molenkampi* eggs was shown in Table 1. The sensitivity and specificity of this method to differentiate *O. viverrini* eggs from *H. taichui,* and *P. molenkampi* eggs were 95% and 95%, respectively.

DISCUSSION

The appearance of the eggshell surfaces was similar to those demonstrated by using a scanning electron microscope (Ditrich *et al*, 1990, 1991; Tesana *et al*, 1991).

In contrast to the scanning electron microscope, a light microscope is more practical. Comparative studies on the eggshell surfaces of fluke eggs using a light microscope have been reported. Temporary stains such as iodine stain (Kaewkes *et al*, 1991) and potassium permanganate stain (Sukontason *et al*, 1999) have been used to demonstrate the eggshell surfaces. Since

methylene blue is commonly used as a simple stain for microorganisms and has a long life on the shelf after prepared, it can be an alternative to stain detailed surfaces on fluke eggshells.

The methylene blue stain for identification of *O. viverrini* eggs had higher sensitivity, specificity, and accuracy when comparing to those used for identification of *H. taichui* eggs. The ridges on *O. viverrini* eggs were higher and more prominent than those found in *H. taichui* eggs (Tesana *et al*, 1991).

In summary, the data presented herein recommended that the methylene blue staining method provided a means for distinguishing *O. viverrini* eggs from minute intestinal fluke eggs in stool examination. This method is more desirable and available in the field, particularly for mass fecal examination. Determining the correct prevalence and intensity of *O. viverrini* infections could be helpful in designing a prevention and control program.

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