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NYLON MESH FILTRATION TECHNIQUE FOR QUANTITATIVE DETECTION OF HELMINTH EGGS

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Abstract: Description was given on the material and procedure of the nylon mesh filtration technique for quantitative detection of helminth eggs in the feces, and comparison was made between the filtration and the Kato-Katz techniques on the clearness and preservation of detected eggs and the efficiency of detection. In the filtration technique, fecal matter is filtered through nylon mesh to concentrate the eggs and the eggs are sealed in the mounting medium by melting the mesh fibers. This technique can detect all the helminth eggs including hook worm and small *Clonorchis* eggs that are difficult to be recognized by the Kato-Katz technique. The rate of coincidence of the positive and negative slides of *Schistosoma* eggs between the two techniques was 88.7%, being statistically high. The filtration technique can detect 2.4 times number of eggs that are collected by the Kato-Katz technique, and the sealed eggs are clear enough to identify and can be preserved for months. This filtration technique, simple and suitable for field use, can contribute to find the helminth infections and to evaluate the control work even in developing countries.
Key words: Nylon mesh filtration technique, Kato-Katz technique, Helminth egg, *Schistosoma japonicum*, *Ascaris lumbricoides*, China

INTRODUCTION

The widely used techniques for the detection of helminth eggs are classified into 1) direct smear, 2) flotation and 3) sedimentation techniques (Garcia and Ash, 1979; Ash and Orihel, 1987).

The direct smear technique (WHO, 1994a) is a simple procedure that prepares the fecal specimens by mixing a small amount (about 2 mg) of fecal material with a drop of physiological saline or tap water. This technique is easy to use, but is not good for the quantitative detection of helminth eggs. More fecal material (60-70 mg) can be processed in the original Kato thick smear technique (Kato and Miura, 1954; Zaman and Keong, 1982), in which a cellophane strip soaked in glycerol and malachite green is placed on a sample of feces and the preparation is left until the glycerol has cleared the fecal material to enable helminth eggs to be seen. The Kato-Katz technique (WHO, 1994c), one of modifications of the original Kato thick smear technique, is widely used particularly in schistosomiasis control work. The feces are first sieved through the screen to remove large fecal particles and fill the hole of plastic template to measure amount of feces (20-50 mg). This fecal matter is covered with the cellophane strip soaked in glycerol-malachite green solution and is spread evenly by pressing against another slide glass.

Two types of concentration techniques (flotation and sedimentation) were developed to separate helminth eggs from excess fecal debris through differences in specific gravity. The flotation technique (Ash and Orihel, 1987) concentrates certain helminth eggs using a liquid with a high specific gravity such as saturated sodium chloride (Cheesbrouth, 1987), or zinc sulphate solutions (Ash and Orihel, 1987; Zaman and Keong, 1982). Feces are emulsified in the solution and the suspension is left undisturbed for the eggs to float to the surface, where they can be collected with an inverted cover glass. This technique is useful for light eggs such as hookworm and fertile *Ascaris* eggs but not good for heavy eggs like large trematode, some tapeworm and infertile *Ascaris* eggs.

The sedimentation technique is a procedure for concentrating helminth eggs by sedimenting the eggs under their own weight or by centrifuging. The formalin detergent technique (Cheesbrough, 1987) is a simple sedimentation technique, which uses solution of low specific gravity for sedimenting helminth eggs without centrifuge or facilities. Unfortunately, however, this technique is time-consuming, taking one hour for heavy eggs or overnight for

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lighter eggs to sediment. Formalin (formol) ether technique (WHO, 1994b) is recommended as the best overall technique for concentrating helminth eggs, in which fecal debris and fat are separated by ether after centrifuging the fecal suspension. This technique, however, is suitable for laboratory work with good facilities rather than for field work without electric power.

Among the above-mentioned techniques, the Kato-Katz technique is used for field work to detect mainly schistosome eggs, but the specimens become not so clear enough to detect thin-shelled hookworm and small trematode eggs. Moreover, the specimens are messy and smell bad particularly while examining under the microscope. The nylon mesh filtration technique was devised to improve these defects and to detect easily and quantitatively all the helminth eggs in the feces. The present paper describes the procedures of this nylon mesh filtration technique (abbreviated "filtration technique" hereinafter) and evaluates the technique based on the comparison with the Kato-Katz technique.

MATERIALS AND METHODS

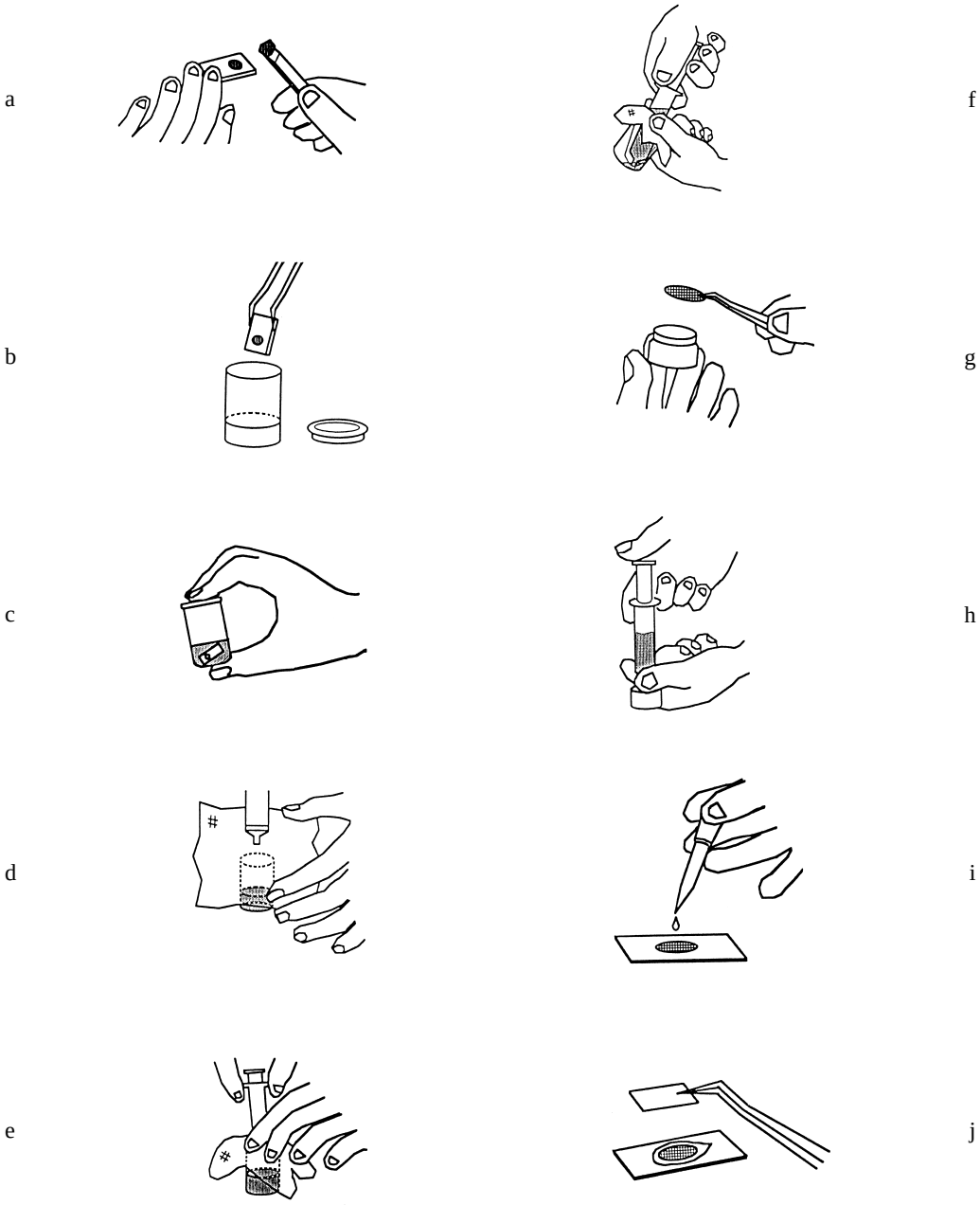
A. *Description of the filtration technique*

1) Materials and reagents

1. Nylon mesh disk (prepared by punching nylon mesh sheet, N-NO. 380T, NBC Kogyo, Tokyo, Japan using leather punch): diameter 25 mm, square opening size 32 μm (length of the diagonal: 45 μm) for *Sch. japonicum* eggs.
2. Membrane filter adapter: for 25 mm membrane filter (Swinex W/O Filter, Cat. No. SX0002500, Millipore Co., Bedford, MA 01730, USA).
3. Plastic syringe: 10 m*l* (Terumo, Cat. No. SS-10S2138, without needle; reusable after washing and lubricating inside with detergent).
4. Applicator stick: 70 mm long×8 mm wide×3 mm thick polypropylene spatura with sharp edges (ordinary wooden matchstick or tooth pick can be used).
5. Organdy screen cloth: 12×12 cm (approximately 100 mesh, hole size 160-200 μm, Spark Tufter, Dandic No. 3023, Uno Co., Japan).
6. Plastic template: 24 mm long×15 mm wide×3 mm thick polypropylene template with a hole of 8 mm diameter bored at 1/3 distance from the template edge {an average of 206 mg feces (N=73, SD=29 mg) can be delivered when the hole is filled completely}.
7. Microscope slide glass: 75×25 mm.
8. Microscope cover slip: 32×24 mm Cut piece (50×24 mm) of OPP (oriented polypropylene, Sekisei Co. Ltd. Cat. No. AZ-575L, Tokyo, Japan) is more convenient than the ordinary microscope cover glass.
9. Roll film case: ordinary marketed size of 5 cm high, 3 cm in diameter (volume ca. 35 m*l*).
10. Forceps.
11. Chloral hydrate solution (prepared by adding 35 g of chloral hydrate, 2 m*l* of glycerol and 0.20 m*l* of saturated eosin solution to 5 m*l* of distilled water and mixing well).
12. 0.5% Tween 80 solution.
13. Used newspaper.

2) Procedure

1. Place a small mound of feces and a plastic template on used newspaper.
2. Collect some amount of feces with an applicator stick and fill the template hole completely. Remove excess feces from the edge of the hole with the applicator stick (Fig. 1-a).
3. Pour about 10 m*l* of 0.5% Tween 80 solution or water into a film case (one fourth of the film case volume measures about 10 m*l*).
4. Put the template with feces in the film case (Fig. 1-b) and close the cap of film case tightly.
5. Shake vigorously the film case lengthwise (Fig. 1-c) so that material is mixed with 0.5% Tween 80 solution or water to become uniform emulsion (Twenty or more times of shaking is necessary for ordinary fecal samples. If the fecal matter is too viscous to mix, mash it with an spplicator stick).
6. Open the film case and take the template out of the case.
7. Cover the film case with a piece of Organdy screen so that the center of the screen comes to the mouth of the film case (Fig. 1-d).
8. Push the Organdy screen with a syringe up to the bottom of the film case so that tip of the syringe can reach every corner of the bottom (Fig. 1-e).
9. Shake the film case sideways and suck up quickly all the emulsion with the syringe. It is easy to suck up all the emulsion by slanting and rotating the film case slowly while sucking so that the debris do not stuff mouth of the syringe (Fig. 1-f).
10. Place a piece of nylon mesh disk on the membrane filter adapter and close adapter cover tightly (Do not use the gasket so that the excess air can leak) (Fig. 1-g).
11. Connect the syringe to the adapter and filter the fecal emulsion through the nylon mesh disk (Fig. 1-h).
12. If time is enough, pour about 10 m*l* of tap water into the film case and suck up all the fecal emulsion again in the same manner as the step 9.
13. Pass air through the mesh disk to drive out excess



a The hole of a template is filled with feces with an applicator.
b The template is put in a film case with feces.
c Fecal material is separated and mixed to become emulsion by shaking the film case.
d The film case is covered with Organdy screen.
e The Organdy screen is pushed by a syringe up to the bottom of the film case.
f The fecal emulsion is sucked through the Organdy screen.
g A nylon mesh disk is put on the membrane filter adapter.
h The fecal emulsion is filtered through the nylon mesh.
i Mounting medium is dropped onto the mesh disk.
j Nylon mesh disk is covered with a cover slip.

Figure 1 Procedure of the nylon mesh filtration technique.

water from the disk.

14. Open the membrane filter adapter and shift the nylon mesh disk onto a slide glass by using forceps.
15. Drop enough amount {10 drops with Pasteur pipette (=ca. 0.4 m*l*) or more} of the chloral hydrate solution onto the mesh disk (Fig. 1-i) and spread the solution together with debris all over the mesh disk.
16. Cover the nylon mesh disk with a cover slip carefully so that the mesh disk comes to the center of the slide glass (Fig. 1-j).

3) Comments and options

Membrane filter adapter, plastic syringe, plastic template, applicator stick and roll film case can be reused after washing completely to avoid contamination. When necessary to disinfect the fecal materials, formalin is added to 0.5% Tween 80 solution or water to reach 1-5% formalin concentration.

It takes about 4 minutes on average to prepare one slide by beginners using this technique. In order to minimize time, the step 12 in the procedure can be skipped. The nylon mesh disk begins to melt soon and disappears under the cover slip after the specimen is prepared. The helminth eggs become visible clearly after nylon fiber disappears in a few minutes. Even if the nylon mesh cannot melt completely, the helminth eggs can be detected and identified by increasing the light intensity and by forcusing appropriately. The specimen can be preserved for months under room temperature.

The specimens should be kept on the horizontal surface for about one day to dry the mounting medium sufficiently. Delicate care is necessary not to allow the excess medium flow out from the slide glass especially when the specimens are carried on the rough road. Nylon mesh with different opening size (20, 25, 32, 37, 42, 48, 53, 59 μm) is available commercially for targeted egg sizes of helminths.

Some weak or premature eggs of *Ascaris lumbricoides* may slightly be distorted in the chloral hydrate solution, but there is no practical problem for detecting and identifying the eggs. In order to avoid the distortion, another chloral hydrate solution (a mixture of 8 g of chloral hydrate, 5 m*l* of glycerol, 10 m*l* of m-cresol, 10 drops of saturated eosin solution and 5 m*l* of distilled water) can be used. Chloral hydrate solution has slightly acid odor and attention should be paid to dry the mounting medium sufficiently before examination.

B. *Comparison between the filtration and the Kato-Katz techniques*

A comparison was designed between the filtration and the Kato-Katz techniques by examining the fecal specimens taken from the same fecal samples. This field experiment was carried out in November 1999 in Gaofeng Village, an endemic area of schistosomiasis japonica in Ruichang City, Jiangxi Province, People's Republic of China. One set of the some 100 fecal slides prepared by the filtration technique was brought to Japan and helminth eggs were identified and counted carefully under a microscope. Another set of the 100 slides prepared by the Kato-Katz technique were examined by the microscopists of the Jiangxi Provincial Institute of Parasitic Diseases (JPIPD). Two sets of the field data from the different techniques were analyzed statistically using VisualStat, Statistica 5j (StatSoft Inc.).

RESULTS

1. Comparison of the clearness and preservation of helminth eggs in the specimen between the filtration and the Kato-Katz techniques

Photographs 1-a and 1-b are *Sch. japonicum* eggs detected by the filtration and the Kato-Katz techniques, respectively. As can be seen in the photographs, *Sch. japonicum* egg is much clearer in the slide made by the filtration than that by the Kato-Katz techniques. Anterior part and outline of a miracidium are visible within the egg shell in the former specimen.

Photographs 2-a and 2-b are *Asc. lumbricoides* eggs detected by the filtration technique and the Kato-Katz technique, respectively. *Asc. lumbricoides* eggs can easily be differentiated from *Sch. japonicum* eggs in the filtration preparation by clear egg shell stained with eosin.

Photograph 3-a and 3-b are *Trichuris trichiura* eggs detected by the filtration techniques and the Kato-Katz, respectively. The egg can be seen more clearly by the former than the latter technique.

Photograph 4 is a hookworm (*Ancylostoma* sp.) egg detected by the filtration technique. This kind of thin-shelled and transparent egg is easily missed or unrecognizable in the Kato-Katz specimen. Photograph 5 is a *Clonorchis sinensis* egg detected by the filtration technique. Small eggs like this trematode egg are very difficult to find out by the Kato-Katz technique but easily recognized by the filtration technique.

The helminth eggs in the filtration technique are sealed with nylon coat which is melted by chloral hydrate or m-cresol in the above-mentioned mounting mediums. These nylon-laminated eggs can be preserved for longer time than those in the Kato-Katz technique. Most of the eggs were preserved for months and clear enough to identify.

2. Comparison of the efficiency to detect helminth eggs between the filtration and the Kato-Katz techniques

It was elucidated by the filtration technique that 36 (37.1%) of 97 slides were positive for one species, 4 slides

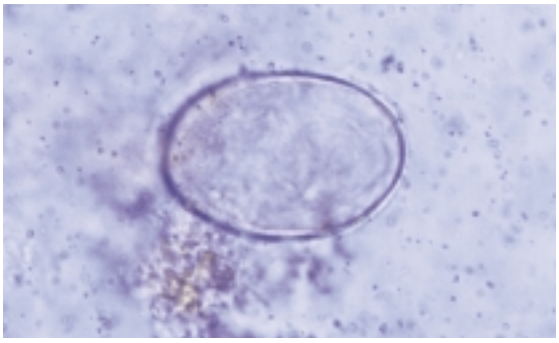


Photo. 1-a

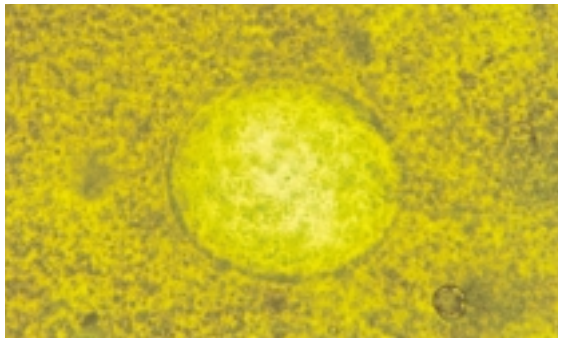


Photo. 1-b

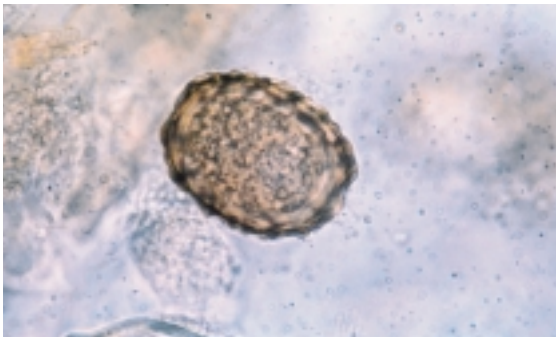


Photo. 2-a

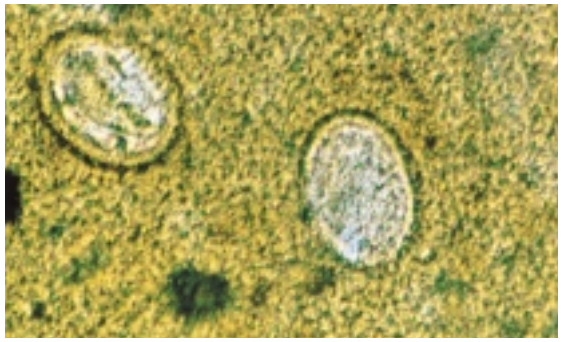


Photo. 2-b

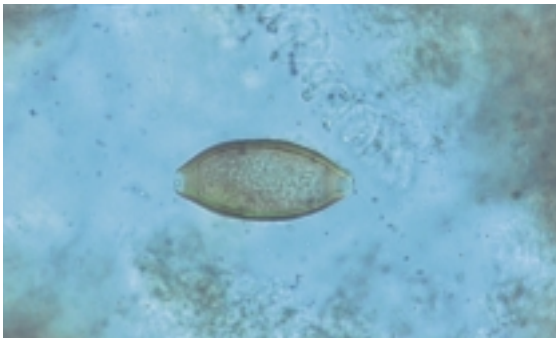


Photo. 3-a

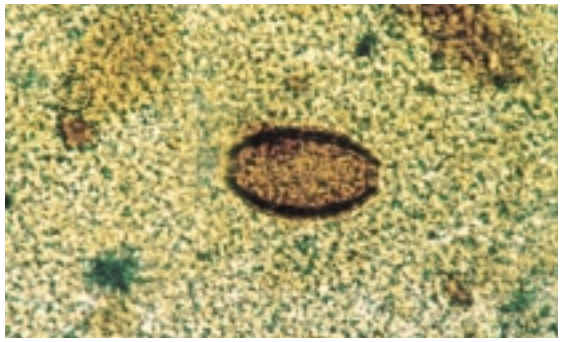


Photo. 3-b

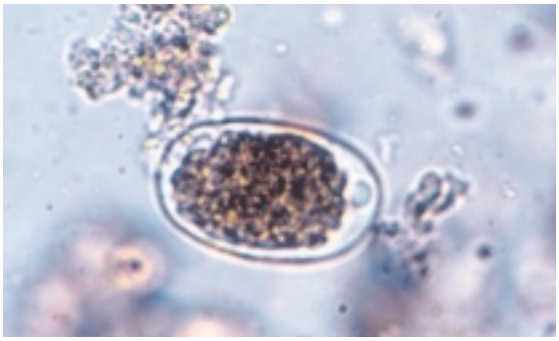


Photo. 4

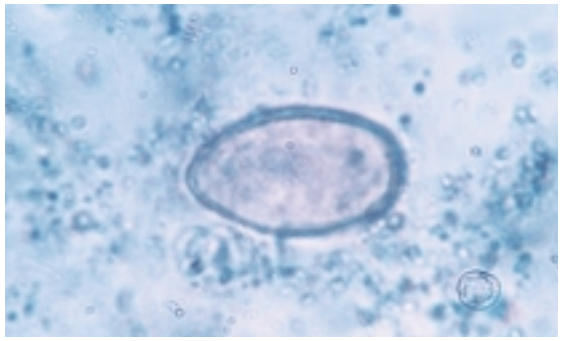


Photo. 5

Photo. 1-a *Schistosoma japonicum* egg detected by the filtration technique.
Photo. 1-b *Sch. japonicum* egg detected by the Kato-Katz technique.
Photo. 2-a *Ascaris lumbricoides* egg detected by the filtration technique.
Photo. 2-b *Asc. lumbricoides* eggs detected by the Kato-Katz technique.
Photo. 3-a *Trichuris trichiura* egg detected by filtration technique.
Photo. 3-b *Tri. trichiura* egg detected by the Kato-Katz technique.
Photo. 4 Hookworm (*Ancylostoma* sp.) egg detected by the filtration technique.
Photo. 5 *Clonorchis sinensis* egg detected by the filtration technique.

(4.1%) for two species and 3 slides (3.1%) for 3 species of helminth eggs. Among these, 33 slides (34.0%) were positive for *Sch. japonicum*, 7 (7.2%) for *Asc. lumbricoides*, 3 (3.1%) for *Trichostrongylus* sp. and one each (4.1% in total) for *Trichuris trichiura*, *Ancylostoma* sp., *Clonorchis sinensis* and *Taenia* sp., respectively. The infection rate with *Sch. japonicum* was higher by the filtration than by the Kato-Katz techniques, being 34.0% (33/97) against 32.0% (31/97). No *Ancylostoma*, *Trichostrongylus* and *Clonorchis* eggs were detected by the Kato-Katz technique.

Among all the 97 slides examined for egg of *Sch. japonicum*, 27 slides were positive and 59 negative in both techniques, 7 were positive by the filtration but negative by the Kato-Katz techniques and 4 were negative by the filtration but positive by the Kato-Katz techniques. As a results, the rate of coincidence between the two techniques was calculated as 88.7% {(27+59)/97×100}, and the McNemar test showed that this rate was significantly high level of coincidence (observed $\chi^2=0.3636$, p=0.5465).

Figure 2 shows the relationship of number of eggs per slide between the Kato-Katz (x) and the filtration (y) techniques. As shown by a high value of coefficient of correlation (r=0.979, p<0.01) based on sufficient number of the fecal samples (n=97), a definite correlation is expected between the two techniques. It is especially noteworthy that the regression line of y on x, y=2.406x-0.789, lies far higher than a line, y=x, along which the number of eggs are expected to be equivalent to each other between the two techniques. It means that the filtration technique is far sensitive in the sense of collecting more eggs than the Kato-Katz technique.

It is possible to convert the number of eggs per sample from the Kato-Katz to the filtration techniques using the above regression equation. For example, a number of 239.8 (95% confidence interval: 229.8-249.8) eggs were expected by the filtration technique against 100 eggs by the Kato-Katz technique from the identical fecal sample (Fig. 2)

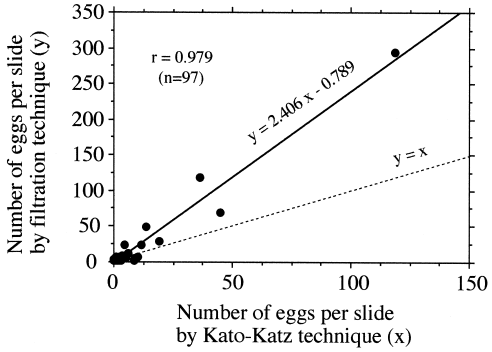


Figure 2 Relationship of number of *Schistosoma* eggs per slide between the Kato-Katz (x) and the filtration techniques (y).

In like manner, the number of eggs is estimated by another regression line of x on y, x=0.398y+0.454, from the filtration to the Kato-Katz techniques. As a result of calculation, a total of 200 eggs by the filtration technique correspond to about 80.1 (95% confidence interval: 76.8-83.4) eggs by the Kato-Katz technique.

The density of egg (eggs per gram: EPG) can be calculated by dividing the total number of eggs by the mean weight of feces (200 mg) in case of the filtration technique. The egg density is important parameter for estimating the worm burden of patients and for evaluating the morbidity rate of a population.

DISCUSSION

Besides above-mentioned parasitological techniques for the detection of helminth eggs, some other techniques have been developed especially for detecting *Schistosoma* eggs in feces of human or domestic animals, i.e. Bell filtration (Bell, 1963), Visser filter (Visser and Pitchford, 1972) and DCEP (Danish Center for Experimental Parasitology) (Willingham *et al.*, 1998) techniques. All of them, however, have the disadvantage that more equipment is required than the Kato-Katz for making specimens in the field (Jordan *et al.*, 1981; Shutte *et al.*, 1994; Willingham *et al.*, 1998).

According to an extensive epidemiological study (Ebrahim *et al.*, 1997), prevalence estimates and mean egg counts revealed the distinct superiority of the Kato-Katz technique (three to four smears) compared with the formol ether sedimentation. They explained this superiority by the sieving effect, in which the quantity of stool in the Kato-Katz technique was measured after the removal of all fibrous and other coarse material. The paradox of low egg count and the diagnostic capability of the formol ether technique was explained by them as follows: Eggs may be lost in the procedural steps of the sedimentation technique despite the fact that more feces is processed (1 g compared with 167 mg/four Kato-Katz smears) and the eggs are easier to distinguish than on Kato-Katz smear.

However, the following disadvantages were pointed out for the Kato-Katz technique: 1) it is “messy”, 2) the eggs are obscured by the thickness of the preparation (Jordan *et al.*, 1981), 3) three or four more smears are necessary to detect light infections (Sleigh *et al.*, 1982; Ebrahim *et al.*, 1997). The present study introduced a new simple technique to improve above-mentioned defects of the Kato-Katz technique. This method can process more (some 200 mg) fecal material than four Kato-Katz smears and eggs are easier to detect than on the Kato-Katz preparation.

In addition to common eggs detected in the endemic area of *Sch. japonicum* in China, the filtration technique

was applied to some other eggs of human and domestic animals using the same or different opening size of nylon mesh. Successful results were obtained for human helminths such as cestodes (*Diphyllobothrium latum, Taenia saginata* and *Echinococcus multilocularis*) and trematodes (*Sch. mansoni, Fasciola hepatica, Paragonimus westermani* and *Metagonimus yokogawai*), and for helminths of domestic animals such as nematodes (*Asc. suum, Toxocara canis, Tox. cati, Tox. vulpis, Tri. suis* and *Ancylostoma caninum*).

The filtration technique was applied to analyze the relationship between the prevalence of hepatic milk spots in pig and the egg density of *Asc. suum.* As a result, a significant correlation was observed between the average egg density and the group prevalence rate of the hepatic milk spots in pig populations. This result proved that this technique can be used as an effective inspection method for detecting milk spot liver in pig (Kano and Makiya, 2001).

As previously mentioned, the flotation technique is useful for some light eggs but not good for heavy eggs like large trematode and some tapeworm eggs. The sedimentation technique is time-consuming or not suitable for field work without electric power. The filtration technique is not only suitable for all kinds of helminth eggs in feces but also for urine-originated eggs like *Sch. hematobium,* in which small aliquot (e.g., 10 m*l*) of urine sample is enough to prepare a slide specimen.

The filtration technique can detect small fecal particles other than helminth eggs, for example, immature nematode larvae such as *Strongyloides stercoralis,* house dust mites like *Dermatophagoides* spp. and their eggs and many kinds of pollens. Actually, a total of 9 mite eggs were detected from 2 fecal samples of the same villagers in the present study. A modification of this filtration technique was utilized for collecting house dust mites and their eggs and juveniles (Makiya, 1999).

The plastic template used in the present study is 3 mm thick with a hole 8 mm in diameter, giving the capacity of 151 mm^3. The capacity of the hole can be increased up to 290 mm^3 by using template 4 mm thick with a hole 9.6 mm in diameter. A regression line, $y=1.528x+1.447$ was obtained between the number of eggs collected by the bigger hole (y) and that by the smaller hole (x) in a preliminary experiment ($n=14$, $r=0.977$, $p<0.01$). This means that some 1.5 times as many as *Sch. japonicum* eggs were collected by the template with a bigger hole than by that with a smaller hole. As a result, it is expected that some 3.7 times ($=2.406\times1.528$) more *Schistosoma* eggs can be collected by the filtration than by the Kato-Katz techniques using this bigger hold template.

The detected eggs are clear enough to recognize and identify despite the fact that the fecal matter is almost doubled in the bigger hole. This new template is recommended to use in order to increase the detection efficiency when the egg density of *Schistosoma* decreased after control measure in future.

In this technique, cut piece (50×24 mm) of OPP (oriented polypropylene) sheet is recommended instead of cover glass to cover the fecal specimen. This cheap material is plastic and light (specific gravity ca. 0.9, weight 64.8 mg/piece against 280 mg/piece of cover glass), and suitable for adding more mounting medium afterwards if the fecal specimen dries. The nylon mesh disk, an important material for the filtration technique, costs only one-tenth (ca. 20 Japanese Yen or 0.16 US $/sheet) of membrane filter that is often used for the same purpose.

It can be concluded from these facts that the filtration technique is "all-inclusive" and cheap technique suitable for detecting all the helminth eggs in the field even where good facilities are not available. Considering the background that most of helminth infections are distributed in developing countries with financial problem, this filtration technique can contribute to find helminth infections and evaluate the control work in such areas.

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REFERENCES

1) Ash, L.R. and Orihel, T.C. (1987): Examination of fresh and preserved feces. *In* "Parasite: A guide to laboratory procedures and identification", 15-35, American Society of Clinical Pathologists Press, Chicago.
2) Bell, D.R. (1963): A new method for counting *S. mansoni* eggs in faeces. Bull. W.H.O., 29, 525-530
3) Cheesbrough, M. (1987): Techniques used to identify

parasites. *In* “Medical laboratory manual for tropical countries”, 178-198, Eng. Lang. Book Soc., Butterworths.

4) Ebrahim, A., El-Morshedy, H., Omer, E., El-Daly, S. and Barakat, R. (1997): Evaluation of the Kato-Katz smear and formol ether sedimentation techniques for quantitative diagnosis of *Schistosoma masoni* infection. Am. J. Trop. Med. Hyg., 57, 706-708

5) Garcia, L.S. and Ash, L.R. (1979): Macroscopic and microscopic examination of fecal specimens. *In* “Diagnostic parasitology, clinical laboratory manual”, 9-18, The C. V. Mosby Co. St. Louis.

6) Jordan, P., Bartholomew, R.K. and Peters, P.A.S. (1981): A community study of *Schistosoma mansoni* egg excretion assessed by the Bell and a modified Kato technique. Ann. Trop. Med. Parasitol., 75, 35-40

7) Kano, S. and Makiya, K. (2001): Relationship between the prevalence of hepatic milk spots in pig and the egg density of *Ascaris suum* in Kitakyusyu Meat Inspection and Control Center (in Japanese with English summary). J. Univ. Occupat. Enviroment. Hlth., 23, 255-262

8) Kato, K. and Miura, M. (1954): Comparative examinations (in Japanese). Parasitol., 3, 35

9) Makiya, K. (1999): Monitoring and its evaluation (in Japanese). *In* “Sanitary management of rats and medically important insects (Series of Environmental and Sanitary Management Technology)”, 268-280, Fuji Technosystem, Tokyo.

10) Shutte, C.H.J., Pienaar, R., Becker, P.J., Viljoen, E., Growther, G., Van Deventer, J.M.G. and Markus, N.B. (1994): Observations on the techniques used in the qualitative and quantitative diagnosis of schistosomiasis. Trop. Med. Parasitol., 3, 305-316

11) Sleigh, A., Hoff, R., Mott, K., Barreto, M., De Paiva, T, Pedrosa, J.S. and Sherlock, I. (1982): Comparison of filtration staining (Bell) and thick smear (Kato) for the detection and quantitation of *Schistosoma masoni* eggs in faeces. Trans. Roy. Soc. Trop. Med. Hyg., 76, 403-406

12) Visser, P.S. and Pitchford, R.J. (1972): A simple apparatus for rapid recovery of helminth eggs from excreta with special reference to *Schistosoma mansoni*. South African Med. J., 46, 1344-1346

13) WHO (1994a): Direct fecal smears - saline and iodine wet mount preparations. *In* “Bench aids for the diagnosis of intestinal parasites”, Plate l, Geneva.

14) WHO (1994b): Fecal concentration procedure - formalin-ether/ethyl acetate/gasolin. *In* “Bench aids for the diagnosis of intestinal parasites”, Plate 2, Geneva.

15) WHO (1994c): Kato-Katz technique - cellophane fecal thick smear. *In* “Bench aids for the diagnosis of intestinal parasites”, Plate 3, Geneva.

16) Willingham, A.L., Johansen, M.V. and Barnes, E.H. (1998): A new technic for counting *Schistosoma japonicum* eggs in pig feces. Southeast Asian J. Trop. Med. Public Health, 29, 128-130

17) Zaman, V. and Keong, L.A. (1982): Basic diagnostic techniques. *In* “Handbook of Medical Parasitology”, 195-215, AIDS Health Science Press, New York.

EFFECTS OF MINOCYCLINE AGAINST MEFLOQUINE-, CHLOROQUINE- AND PYRIMETHAMINE-RESISTANT *PLASMODIUM FALCIPARUM IN VITRO*

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Abstract: Tetracyclines are used for prophylaxis of malaria and treatment of drug-resistant falciparum malaria because of their safe drug action. We re-evaluated effects of three tetracyclines against drug-resistant *Plasmodium falciparum in vitro*. Minocycline was approximately 4 times and twice more potent in inhibiting the *in vitro* growth of falciparum parasites than tetracycline and doxycycline, respectively. Compared with doxycycline, significant inhibitory effects of minocycline to chloroquine, pyrimethamine and mefloquine resistant *P. falciparum* strains were affirmed by the present *in vitro* study. By electron microscopy a number of electron dense vesicles with a single membrane bound were observed in the cytoplasm of minocycline-treated parasites, although no distinct structural alternations of mitochondria was noted. Minocycline may be a better therapeutic drug than doxycycline which is widely accepted as the standard antimalarial tetracycline.

Key words: *Plasmodium falciparum*, Drug-resistance, Electron microscopy, Tetracyline, Minocycline, Chloroquine, Mefloquine, Pyrimethamine

INTRODUCTION

Chemotherapy is the primary defense against malaria. Therefore, the spread of drug resistant *Plasmodium falciparum* is a world-wide threat. Development of new antimalarials is one of the major goals of malaria research. However, development and deployment of a new drug is extremely expensive, causing discouragement for pharmaceutical companies to search for new therapeutic agents. This leads to re-evaluation of antimalarial activities of any drugs which have already been accepted for clinical use in patients with various infectious diseases other than malaria. Antibiotics are one of the major sources for such trials. Amongst commonly used antibiotics, a group of tetracyclines has been most widely used for treatment of malaria (Puri and Dutta, 1982). The effect of chlortetracycline against a malaria parasite was first reported by Coatney (Coatney *et al.*, 1949). Since then, tetracyclines have been tried in malaria treatment (Pang *et al.*, 1987; Looareesuwan *et al.*, 1992; Rieckman *et al.*, 1971). The conventional tetracycline is a short acting agent (plasma half-life: 8.5 hr), while doxycycline and minocycline have relatively long half-lives (plasma half-life: 18-22 hr and 12-16 hr, respectively (Dollery, 1999), permitting better selection of these tetracyclines for malaria treatment than tetracycline. Doxycycline in particular, is accepted as an antimalarial for chemotherapy as well as chemoprophylaxis (Colwell *et al.*, 1972; Shanks *et al.*, 1992). The present study focused on the potency of the antimalarial activity of minocycline to cultured drug-resistant *P. falciparum* parasites in comparison with tetracycline and doxycycline. We show here the first report that minocycline was effective against mefloquine-resistant falciparum parasites *in vitro*. The presumed molecular mechanisms of tetracyclines and the reason for the highest potency shown by minocycline are discussed.

MATERIALS AND METHODS

Parasites

Two established strains and two recent clinical isolates of *P. falciparum* were used in the present study (Table 1). SGE-1 is a drug sensitive strain of Gambian origin, donated

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by Dr. P. Ambroise-Thomas of the University of the Grenoble in 1979, and has been maintained by *in vitro* culture alternating occasional freezing in liquid nitrogen in our laboratory. Regardless of long-term cultivation, the parasite has maintained its virulence, causing fatal sickness in *Aotus* monkeys. K-1 is a chloroquine-resistant strain isolated in Thailand, which was donated by the London School of Hygiene and Tropical Medicine in 1984. One isolate, MZG, is a chloroquine-sensitive but mefloquine-resistant parasite isolated from a Japanese patient who developed falciparum malaria after returning from Mozambique in 1998. Another isolate NGG is a moderate chloroquine-resistant and highly pyrimethamine-resistant parasite obtained from a Japanese patient who also developed falciparum malaria after visiting Nigeria in 1997.

Cultivation of P. falciparum parasites and in vitro drug susceptibility test

Culture of falciparum parasites was carried out according to a modified method of Trager and Jensen (Trager and Jensen, 1976), using RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Japan) medium with 10% human serum {RPMI 1640 (+)} and type O human red blood cells. The drug susceptibility test was performed by a semi-micro method, described previously (Bras and Deloron, 1983; Inaba *et al.,* 2001). Parasites were synchronized by D-sorbitol treatment (Lambros and Vanderberg, 1979), and parasitized erythrocytes with a 0.15-0.3% infection rate were adjusted to a 5% packed cell volume in RPMI 1640 (+) at the start of incubation. The test was done using 24-well plates (Falcon, 3047, Becton Dickinson, NJ, USA) and each well contained 500 μl of parasitized erythrocyte suspension and 20 μl of drug solution containing the respective concentrations of each drug. Final drug concentrations of tetracyclines were 0, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100 and 300 μg/ml. The incubator was kept at 37℃ continuously with a gas flow mixture composed of 5% O_2, 5% CO_2 and 90% N_2. The medium was changed every day and the cultivation was continued for up to 4 days (Divo *et al.,* 1985; Geary and Jensen, 1983; Inaba *et al.,* 2001). The effects of drugs on the growth of the parasites were expressed by the 50% inhibitory concentrations (IC_{50}), which were calculated by computerized probit analysis.

Drugs used and the sources were as follows: tetracycline hydrochloride and minocycline hydrochloride from Lederle Ltd., Japan; doxycycline from Sigma Chemical Co., USA; chloroquine sulfate from Winthrop Stearns Inc., Manila, Philippines; mefloquine from Roche Diagnostics, Switzerland; pyrimethamine from Wako Pure Chemical Industries, Ltd., Japan.

Electron microscopy

Erythrocytes infected with SGE-1 parasites were treated with tetracycline at 1.0 μg/ml and minocycline at 0.3 μg/ml, approximately at the IC_{50} values described in the text. Blood samples were taken every 24 hr after drug exposure and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4℃ for 2 hr (Kawai *et al.,* 1996). The specimens were postfixed in 1% osmium tetroxide for another 2 hr, dehydrated in a graded alcohol series, treated with propylene oxide, and embedded in Epon 812. The resultant blocks were cut with a Porter Blum (Newton, CT) MT-2 ultramicrotome with a Diatome (Bienne, Switzerland) diamond knife. Thin sectioned specimens were mounted on 200-mesh copper grids, stained with uranyl acetate plus lead citrate. The prepared specimens were observed using a transmission electron microscope (Hitachi, H-800, Tokyo, Japan).

RESULTS

Drug resistant profiles of P. falciparum parasites used in the present study

Drug susceptibilities of three *P. falciparum* parasites were examined by a semi-micro *in vitro* drug susceptibility test, in comparison with SGE-1 as a standard strain (Table 1). The IC_{50} of K-1 for chloroquine was 4.4-fold higher than that of the SGE-1 strain. The K-1 strain also showed

Table 1 Drug susceptibility of *P. falciparum* isolates

Strain	IC_{50}^{*}		
	Chloroquine (μM)	Mefloquine (nM)	Pyrimethamine (nM)
SGE-1	0.086±0.002	13.97±2.64	69.1±7.93
K-1	0.378±0.019	12.78±3.85	126±10.86
MZG	0.040±0.001	248.3±50.17	75.8±8.52
NGG	0.22±0.017	4.88±0.89	2,774±109.4

* Values are mean±SD for three experiments

SGE-1 was used as a standard *P. falciparum* strain which showed susceptibilities to chloroquine, mefloquine and pyrimethamine. K-1, MZG and NGG strains showed selective resistance to chloroquine, mefloquine and pyrimethamine respectively.

moderate resistance to pyrimethamine. The clinical isolate, MZG of Mozambique origin, showed a 17.8-fold higher IC_{50} for mefloquine. Another clinical isolate, NGG of Nigerian origin, was highly resistant to pyrimethamine with a 40-fold higher IC_{50} than that of the SGE-1 strain. These findings confirmed that all strains and isolates of falciparum parasites used in the present study had maintained their stable phenotypes in terms of original drug sensitivities to each antimalarial drug.

Comparison of effects of three tetracyclines on the growth of P. falciparum parasites in vitro

We compared the inhibitory effects of tetracycline, doxycycline and minocycline on the standard SGE-1 strain by determining the IC_{50} values at 24, 48, 72 and 96 hr after drug exposure. The antimalarial activities of all three tetracyclines were enhanced when drug exposure was increased from 24 to 96 hr (Table 2), as reported previously (Divo *et al.*, 1985; Geary and Jensen, 1983). However, the inhibitory effect of minocycline was the most potent at each time point examined (Table 2). The IC_{50} value for minocycline at 24 hr was 3.90 μg/m*l*, while those for doxycycline and tetracyline were 10.11 and 17.20 μg/m*l*, respectively. At 96 hr the IC_{50} for minocycline (0.24 μg/m*l*) was 2.1-fold less than that for doxycycline (0.50 μg/m*l*) and 5.1-fold less for tetracycline (1.23 μg/m*l*). Overall, the mean IC_{50} value at different time points for minocycline was approximately 4-fold and 2-fold lower than that for tetracycline and doxycycline.

The efficacy of minocycline was further studied using drug-resistant parasites in comparison with tetracycline. Growth inhibition of parasites was assayed in the presence of tetracycline at 1.0 μg/m*l* or minocycline at 0.3 μg/m*l*, approximating the IC_{50} values, according to the above findings. All falciparum parasites, such as SGE-1, chloroquine-resistant K-1, mefloquine-resistant MZG and pyrimethamine-resistant NGG, showed substantially similar susceptibilities to both tetracycline and minocycline (Table 3). The inhibitory effects of these antibiotics were notable at 72 hr with 20-30% growth inhibition. Eventually, the effect reached to

Table 2 Comparison of effects of three tetracyclines on the growth of *P. falciparum*

Drug	IC_{50} (μg/m*l*) at time points			
	24 hr	48 hr	72 hr	96 hr
Tetracycline (n=5)	17.2±1.52	4.36±0.91	2.26±0.38	1.23±0.24
Doxycycline (n=2)	10.11±1.84	2.45±0.14	1.10±0.01	0.50±0.13
Minocycline (n=5)	3.90±1.60	2.00±0.25	0.85±0.22	0.24±0.01

By *in vitro* comparative study on tetracyclines on SGE-1 strain, it is remarked that minocycline showed 4.4 times higher anti-malarial activity than that shown by doxycycline at 24 hr test. It is notable that minocycline shows inhibitory effect to the parasites at the early stage of the test.

Table 3 Inhibitory effects of tetracycline and minocycline on the growth of drug-resistant *P. falciparum*

Drug*	strain	% Inhibition of growth at time points †			
		24 hr	48 hr	72 hr	96 hr
MC	SGE-1	0	13	31	68
	K-1	-12	16	28	72
	MZG	0	14	33	69
	NGG	7	15	30	65
TC	SGE-1	-9	26	31	44
	K-1	-6	2	31	48
	MZG	4	10	30	40
	NGG	-20	2	17	43

＊ TC, tetracycline at 1.0 μg/m*l*; MC, minocycline at 0.30 μg/m*l*.

† Inhibitory effect was determined by comparison of the parasite growth of untreated and drug-treated parasites in triplicate experiments.

By 24 hr test with low dose tetracyclines, no effect was remarked. Percent growth inhibitory values were equally elevated at high levels at 96 hr test both in the standard strain and resistant strains. Still, MC effect> TC effect is also noted.

the maximum at 96 hr, resulting in 40-48% inhibition by tetracycline and 65-72% by minocycline.

Ultrastructural changes of parasites treated with tetracyclines

The structural alterations of parasites caused by treatment with tetracycline at 1.0 μg/m*l* and minocycline at 0.3 μg/m*l* were examined at 24, 48, 72 and 96 hr after treatment. Distinct differences in the structural changes were not observed between parasites treated with tetracycline and minocycline so far as the present study concerns. Enlargement of the perinuclear cisterna space was noted in the specimen at 24 hr of tetracycline treatment, compared with untreated parasites (Fig. 1A, B and C). A dilatation of cisternae of endoplasmic reticulum was also noted at 24 hr and 48 hr (data not shown). In parasites exposed to tetracyclines for 72 hr, a number of electron dense vesicles with a single membrane bound were observed and the cytoplasmic structure was largely disintegrated (Fig. 1D). However, distinct structural alterations of the organelles, such as mitochondria and plastids, were not noted in the present study.

DISCUSSION

The present study showed that in a group of tetracyclines, minocycline was the most potent for suppressing the growth of cultured *P. falciparum*. The IC_{50} values of tetracyline, doxycycline and minocycline for the *P. falciparum* SGE-1 strain were 1.23, 0.50 and 0.24 μg/m*l*, respectively. Acute systemic toxicity studies showed that the LD_{50} value of these drugs, when orally administered, was 2,000-3,000, 1,700 and 1,900-3,600 mg/kg of body weight, respectively (Dollery, 1999; personal communications from Lederle Japan and Pfizer Japan). When simply comparing the ratio of the LD_{50} versus the IC_{50}, the ratio of minocycline was estimated to be 7,900-15,000, whereas the ratio of tetracycline was 1,600-2,400. This finding indicates that minocycline appears to be 3.3-9.4-fold more potent than tetracycline.

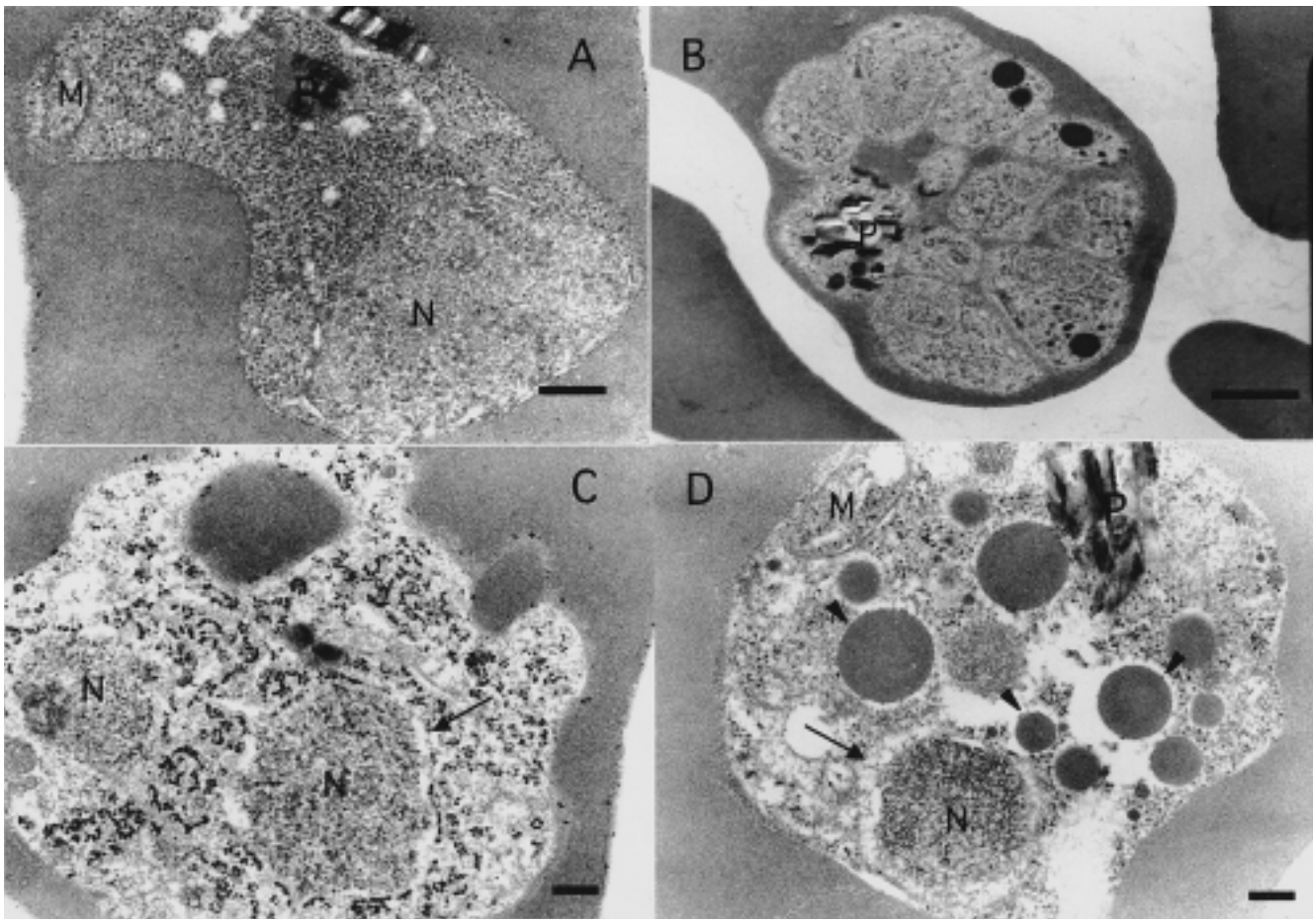

Figure 1 Electron micrograph of *P. falciparum* treated with tetracyclines. An early trophozoite (A) and schizont (B) are shown as a control. Early trophozoites were treated with tetracyclines for 24 hr (C) and 72 hr (D). Dilatations of perinuclear cisterna space are indicated by arrows. Electron dense vesicles are indicated by arrow heads. N, nucleus; M, mitochondrion; P, pigment. Bar=0.2 μm

The index of minocycline also exceeds that of doxycycline with a value of 3,400.

Minocycline eventually showed killing activity for mefloquine-resistant *P. falciparum* parasites as well as chloroquine- and pyrimethamine-resistant parasites, although this antibiotics required a longer time for an appearance of the inhibitory effect than other antimalarial drugs. As far as we know, this is the first report on the effect of minocycline against mefloquine-resistant *P. falciparum in vitro.* This finding suggests that minocycline should be further studied in clinical cases as an excellent candidate for chemotherapy of drug-resistant malaria, even though there were some clinical trials for chloroquine-resistant malaria using this antibiotics in the early 1970's (Colwell *et al.,* 1972; Willerson *et al.,* 1972). Tetracycline has already been used to treat drug-resistant falciparum malaria, generally in combination with quinine (Looareesuwan *et al.,* 1992; Puri and Dutta, 1982; Rieckman *et al.,* 1971; Watt *et al.,* 1992). Our recent experiments showed that a combination of minocycline with artemether and/or chloroquine resulted in an excellent therapeutic effect without showing recrudescence in mice infected with *P. berghei* and chloroquine-resistant *P. chabaudi* (manuscript in preparation).

The rapid anti-parasite action of minocycline *in vitro* study has also been shown in the present study (Table 2). The inhibitory action shown at the early stage of drug exposure is a key point to evaluate antimalarials. All these results support the use of minocycline as a better tetracycline than doxycycline for the treatment of chloroquine-, pyrimethamine- and mefloquine-resistant falciparum malaria, respectively.

The highly inhibitory effects of minocycline on parasite growth shown in the present study may be attributable to the high lipophilicity of minocycline (Tomas, 1989). Lipophilic features of this antibiotics may permit the high permeability through the plasma membrane, mitochondrial membrane and even plastid membrane. The minocycline may be more efficiently transported from the serum to the parasites in the erythrocytes than the other tetracyclines because of its lipophilic feature. Mitamura *et al.* (2000) showed that serum albumin (SA) derived fatty acid species in the neutral lipid fraction in the serum plays an crucial role in the parasite growth at the erythrocytic stage. It appears that SA derived fatty acid passes through the erythrocytic membrane by some mechanism. The finding will suggest a probable role of lipid as the carrier for lipophilic minocycline to pass through erythrocytic memberane and eventually reach to the parasites.

The ultrastructural alterations, such as the dilatation of perinuclear space and appearance of electron dense single membrane-bound vesicles were marked in falciparum parasites treated with tetracyclines in the present study. The electron dense vesicles were also observed in late trophozoites and schizonts of *P. falciparum* taken from *Aotus* monkeys treated with pyronaridine (Kawai *et al.,* 1996). It was notable that these alterations in the parasites occurred before no change came out in mitochondria, which is considered as the target of teracyclines. However, it is difficult, at present, to explain whether the structural changes resulted from the specific action mechanism of tetracyclines. Further investigations into the morphologic changes focusing on the parasite mitochondria and plastids will be of particular importance in future studies.

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REFERENCES

1) Bras, J.L. and Deloron, P. (1983): *In vitro* study of drug sensitivity of *Plasmodium falciparum*: Evaluation of a new semi-micro test. Am. J. Trop. Med. Hyg., 32, 447-451
2) Coatney, G.R., Greenberg, J., Cooper, W.C. and Trembley, H.L. (1949): Effect of chlortetracycline against *Plasmodium gallinaceum.* Proc. Soc. Exp. Biol. Med., 72, 586-587
3) Colwell, E.J., Hickman, R.J., Intraprasert, L.R. and Tirabutan, C. (1972): Minocycline and tetracycline treatment of acute falciparum malaria in Thailand. Am. J. Trop. Med. Hyg., 21, 144-149
4) Divo, A.A., Geary, T.G. and Jensen, J.B. (1985): Oxygen and time-dependent effects of antibiotics and selected mitochondrial inhibitors on *Plasmodium falciparum* in culture. Antimicrob. Agents Chemother., 27, 21-27
5) Dollery, C. (1999): Therapeutic drugs. Churchill Livingstone., London.
6) Geary, T.G. and Jensen, J.B. (1983): Effects of anitibiotics on *Plasmodium falciparum in vitro.* Am. J. Trop. Med. Hyg., 32, 221-225
7) Inaba, H., Ohmae, H., Kano, S., Faarado, L., Boaz, L., Leafasia, J. and Suzuki, M. (2001): Variation of incubation time in an *in vitro* drug susceptibility test of *Plasmodium falciparum* isolates studied in the Solomon Islands. Parasitol. Int., 50, 9-13
8) Kawai, S., Kano, S., Chang, C. and Suzuki, M. (1996): The effects of pyronaridine on the morphology of *Plasmodium falciparum* in *Aotus trivirgatus.* Am. J. Trop. Med. Hyg., 55, 223-229
9) Lambros, C. and Vanderberg, J.P. (1979): Synchronization

of *Plasmodium falciparum* erythrocytic stages in culture. J. Parasitol., 65, 418-420
10) Looareesuwan, S., Wilairatana, P., Vanijanonta, S., Kyle, D. and Webster, K. (1992): Efficacy of quinine-tetracycline for acute uncomplicated falciparum malaria in Thailand. Lancet, 339, 369
11) Mitamura, T., Hanada, K., Ko-Mitamura, E.P., Nishijima, M. and Horii, T. (2000): Serum factors governing intraerythrocytic development and cell cycle progression of *Plasmodium falciparum.* Parasitol. Int., 49, 219-229
12) Pang, L.W., Limsomwong, N., Boudreau, E.F. and Singharaj, P. (1987): Doxycycline prophylaxis for falciparum malaria. Lancet, i, 1161-1164
13) Puri, S.K. and Dutta, G.P. (1982): Antibiotics in the chemotherapy of malaria. Prog. Drug Res., 26, 197-205
14) Rieckman, K.H., Powell, R.D., McNamara, J.V., Willerson, D.J., Kass, L., Frischer, H. and Carson, P.E. (1971): Effect of tetracycline against chloroquine-resistant and chloroquine-sensitive *Plasmodium falciparum*. Am. J. Trop. Med. Hyg., 20, 811-815
15) Shanks, G.D., Edstein, M.D., Suriyamongkol, V., Timsaad, S. and Webster, H.K. (1992): Malaria chemoprophylaxis using proguanil/dapsone combinations on the Thai-Cambodian border. Am. J. Trop. Med. Hyg., 46, 643-648
16) Thomas, D.E. (1989): Tetracyclines as antiparasitic agents: Lipophilic derivatives are highly active against *Giardia lamblia in vitro.* Antimicrob. Agents Chemother., 33, 2144-2145
17) Trager, W. and Jensen, J.B. (1976): Human malaria parasites in continuous culture. Science, 193, 673-675
18) Watt, G., Loesuttivibool, L., Shanks, G.D., Boudreau, E. F., Brown, A.E., Pavanand, K., Webster, H.K. and Wechgritaya, S. (1992): Quinine with tetracycline for the treatment of drug-resistant falciparum malaria in Thailand. Am. J. Trop. Med. Hyg., 47, 108-111
19) Willerson, D. Jr., Rieckman, K.H., Carson, P.E. and Fischer, H. (1972): Effect of minocycline against chloroquine-resistant falciparum malaria. Am. J. Trop. Med. Hyg., 21, 857-863

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SIMULIUM (SIMULIUM) *WEJI* SP. NOV. (DIPTERA: SIMULIIDAE) FROM THAILAND

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Abstract: *Simulium weji* sp. nov. is described based on the reared females, reared males, pupae and mature larvae collected from Tharn Thong Waterfall in Lampang Province, Thailand. This new species is assigned to the *tuberosum* species-group within the subgenus *Simulium* s. str., and seems to be closely related to *S. rufibasis* Brunetti and four other allied species, reported from India and Thailand, by having a pair of hair clusters on the ventral surface of the seventh abdominal segment of the female, which are, though, much longer and fewer in this new species. This new species seems to be also related to *S. nigrifacies* Datta from India and *S. puliense* Takaoka from Taiwan, by having the similar arrangement of six short pupal gill filaments, but is easily differentiated from these known species by the small cone-shaped tubercles on the head and thoracic integuments.
key words: Black fly, Simuliidae, *Simulium*, Thailand, New species

Recent investigations on simuliid fauna of Thailand have increased the total number of black-fly species up to 40 (Takaoka and Saito, 1996; Takaoka and Adler, 1997; Takaoka and Kuvangkadilok, 1999; Kuvangkadilok and Takaoka, 2000). During our recent surveys of black flies in northern Thailand we found one more new species belonging to the *tuberosum* species-group within the subgenus *Simulium* (*Simulium*), defined by Takaoka and Davies (1996).

This new species is here described based on the reared females, reared males, pupae and mature larvae.

Holotype and most paratype specimens will be deposited at the Natural History Museum (BMNH), London, U. K.

Simulium (Simulium) weji sp. nov.

DESCRIPTION. **Female.** Body length ca. 2.3 mm. ***Head.*** Narrower than width of thorax. Frons black, shiny, widely bare except several dark stout hairs along each lateral margin and a few hairs near antennal base; frontal ratio 1.2-1.4: 1.0:1.2; frons-head ratio 1.0:3.7. Fronto-ocular area (Fig. 1) moderately developed, with round lateral tip. Clypeus black, shiny, widely bare medially except dark stout hairs along each lateral margin and several dark stout hairs near lower margin. Proboscis 0.51-0.55× as long as clypeus. Antenna composed of 2+9 segments, light to dark brown except scape, pedicel and base of 1st flagellar segment yellow. Maxillary palp brownish black, composed of 5 segments with proportional lengths of 3rd, 4th and 5th segments 1.0:1.3:2.7; 3rd segment (Figs. 2 and 3) of moderate size; sensory vesicle medium in size, ellipsoidal, with rugged surface, ca. 0.3× length of 3rd segment, with medium round opening. Maxillary lacinia with 15 or 16 inner and 18 outer teeth. Mandible with ca. 26 inner and 12 outer teeth. Cibarium (Fig. 5) with several minute processes. ***Thorax.*** Scutum blackish brown, shiny, not patterned, moderately covered with recumbent dark-brown pubescence, interspersed with long upstanding dark hairs on prescutellar area; scutum thinly grey-pruinose when illuminated in certain angle of light. Scutellum brownish black, with long and short dark hairs. Postscutellum brownish black, shiny, without hairs. Pleural membrane bare. Katepisternum longer than deep, and bare. ***Legs.*** Foreleg: coxa whitish yellow; trochanter and femur dark yellow; tibia light brown except apical 1/5 dark brown, and outer surface of basal 4/5 pale white and silvery iridescent in certain angle of light; tarsus entirely brownish black; basitarsus, 2nd and 3rd tarsal segments dilated (basitarsus ca. 4.9× as long as its greatest width), with distinct dorsal crest of medium-long hairs. Midleg: coxa brownish black; trochanter light brown with base whitish yellow; femur light brown with apical cap dark brown; tibia light brown except base somewhat yellowish narrowly, with white basal sheen on poste-

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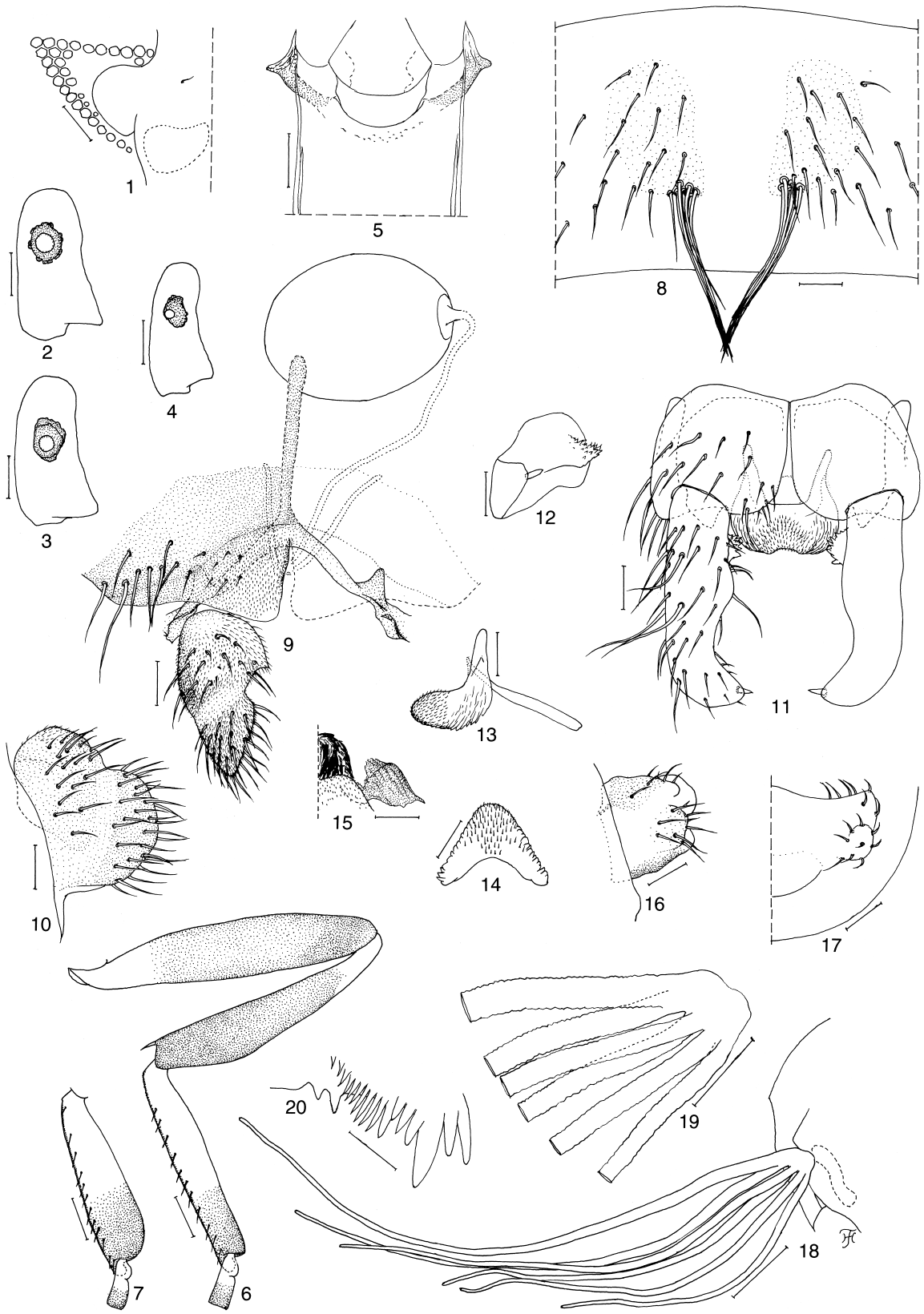
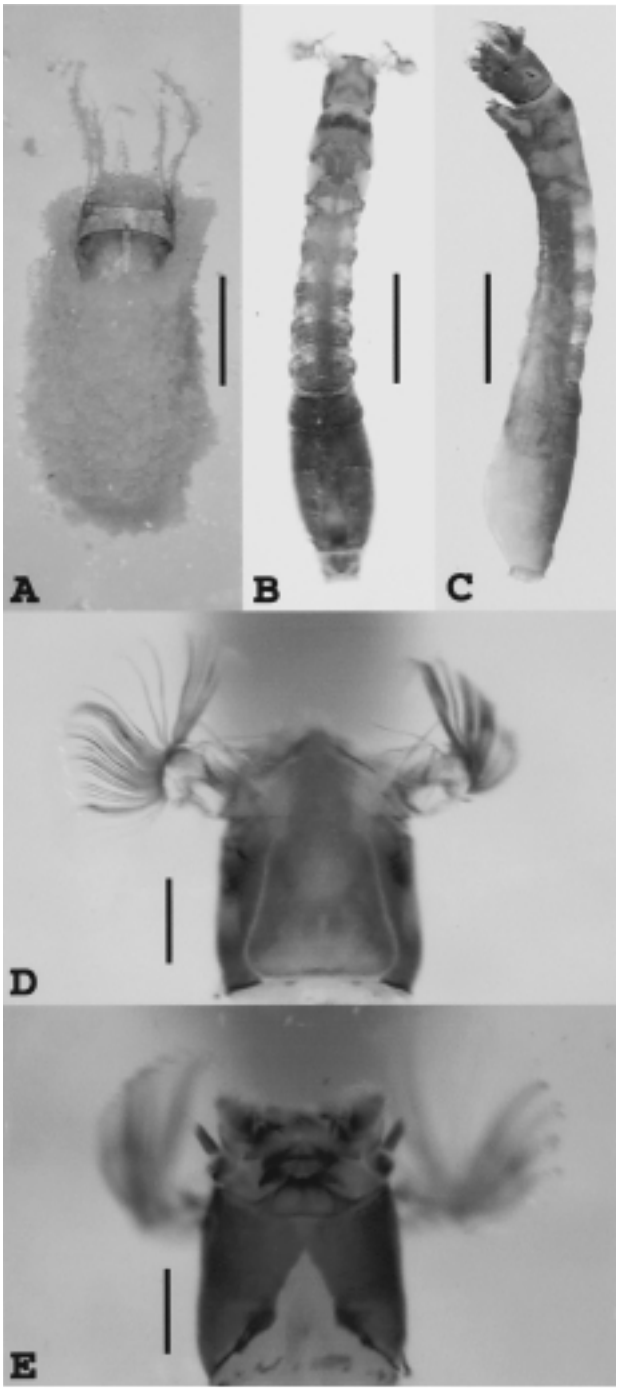
rior surface in certain angle of light; tarsus light brown except basal 4/5 of basitarsus, basal 1/3 or 1/2 of 2nd tarsal segment and base of 3rd tarsal segment whitish yellow, and 5th tarsal segment brownish black. Hind leg (Fig. 6): coxa medium brown; trochanter whitish yellow; femur medium brown with base whitish yellow and with apical cap dark brown; tibia dark brown to brownish black except base whitish yellow, and with large white sheen on posterior surface in certain angle of light; tarsus dark brown except basal 2/3 of basitarsus and basal 1/2 of 2nd tarsal segment whitish yellow; basitarsus nearly parallel-sided, ca. 5.8× as long as its greatest width, and ca. 0.79× and ca. 0.64× as wide as hind tibia and femur, respectively; calcipala short, nearly as long as wide, and ca. 0.4× as wide as greatest width of basitarsus; pedisulcus distinct. All tarsal claws simple, without subbasal or basal tooth. ***Wing.*** Length 1.7-1.9 mm; costa with spinules and hairs; subcosta haired; basal section of vein R bare; hair tuft at base of stem vein dark brown; basal cell absent. ***Abdomen.*** Basal scale brownish black with fringe of long hairs; dorsal surface of 2nd segment pale yellow on anterior 1/2 or 2/3 with a pair of large dorsolateral whitish iridescent spots broadly connected to each other in middle; dorsal surface of posterior 1/2 or 1/3 of 2nd segment and those of other segments dark brown and with dark hairs; tergites 3, 4 and 5 small and dull, tergites 6-8 large and shiny. Ventral surface of abdominal segment 7 with a pair of submedian clusters of 6 or 7 very long stout hairs (their length range 90-140 μm) on the posterior corner of weakly-developed sternal plates (Fig. 8). ***Genitalia*** (Figs. 9 and 10). Sternite 8 well sclerotized, bare medially but with 8-13 short and long stout hairs as well as a few short slender hairs laterally on each side; anterior gonapophysis triangular in shape, membranous, covered with 5 or 6 short hairs as well as numerous microsetae; inner border slightly sinuous, narrowly sclerotized, folded dorsally. Genital fork of inverted-Y form, with well sclerotized stem; arms slender, each with strongly sclerotized apical bulge having a distinct projection directed anteriorly. Paraproct in ventral view nearly as long as wide, with no distinct concavity on ventral surface along anterior margin; anteromedial surface of paraproct strongly sclerotized, and with 5 or 6 short sensilla; paraproct covered with ca. 20 short stout hairs on ventral and lateral surfaces. Cercus in lateral view rounded posteriorly, ca. 0.5× as long as wide, covered with many short hairs. Spermatheca nearly ovoid, well sclerotized with no definite reticulate pattern, with minute internal setae; tube and small area around tubal base unsclerotized; accessary tubes subequal in diameter to each other and to major one.

Male. Body length 1.9-2.1 mm. ***Head.*** Width slightly wider than thorax. Upper eye consisting of large facets in 17 horizontal rows and in 16 vertical columns. Clypeus black, white pruinose, and silvery iridescent when illuminated, widely bare except dark stout hairs along each lateral margin and a few dark stout hairs near lower margin. Antenna composed of 2+9 segments, light to dark brown except 1st, or 1st and 2nd, or 1st to 3rd flagellar segments, as well as scape and pedicel, yellow when viewed ventrally; 1st flagellar segment somewhat elongated, ca. 1.7× as long as 2nd flagellomere. Maxillary palp composed of 5 segments, with its proportional length from 3rd to 5th segments 1.0:1.3:2.6; 3rd segment (Fig. 4) of normal size; sensory vesicle small, ellipsoidal, with small opening. ***Thorax.*** Scutum black, dull, with white-pruinose shiny pattern composed of an anterior pair of triangular spots with rounded apex (a part of which disappears in certain angles of light) on shoulders, each extending posteriorly forming a broad band along each lateral margin up to base of wing, and a large transverse spot entirely covering prescutellar area, which is narrowly contiguous to each lateral band; these pruinose areas silvery iridescent when illuminated in certain angles of light; scutum uniformly covered with dark-brown recumbent pubescences, interspersed with long upright hairs on prescutellar area. Scutellum brownish black, shiny, with several upright dark hairs. Postscutellum brownish black, shiny, without hairs. Pleural membrane and katepisternum as in female. ***Legs.*** Foreleg: coxa whitish yellow; trochanter and femur light brown; tibia medium brown except apical 1/5 dark brown, and outer surface of basal 4/5 narrowly pale white and silvery iridescent in certain angle of light; tarsus entirely brownish black; basitarsus, 2nd and 3rd tarsal segments dilated (basitarsus ca. 4.5× as long as its greatest width), with distinct dorsal crest of medium-long hairs. Midleg: coxa brownish black; trochanter medium brown with base whitish yellow; femur medium brown with apical cap dark brown; tibia medium brown except base narrowly white; tarsus light brown except basal 4/5 or 5/6 of basitarsus, basal 1/3 of 2nd tarsal segment and base of 3rd tarsal segment whitish yellow, and 5th tarsal segment brownish black. Hind leg: coxa dark brown; trochanter whitish yellow; femur dark brown with base whitish yellow and with apical cap brownish black; tibia dark brown to brownish black except base whitish yellow; tarsus (Fig. 7) dark brown except basal 3/5 of basitarsus and basal 1/2 of 2nd tarsal segment whitish yellow; basitarsus (Fig. 7) much enlarged, ca. 3.4× as long as its greatest width at apical l/4, and ca. 0.93× as wide as greatest width of hind tibia which is subequal to that of hind femur. Calcipala small, nearly as long as wide, ca. 0.24× as wide as greatest width of basitarsus. Pedisulcus well marked. ***Wing.*** Length 1.5-1.6 mm; other features as in female except subcosta bare. ***Abdomen.*** Basal scale blackish with fringe of long dark hairs. Dorsal



Figures 1-20. Morphological features of *S. weji* sp. nov. 1, female fronto-ocular area; 2-4, 3rd segment of maxillary palp with sensory vesicle (2 and 3, female; 4, male); 5, female cibarium; 6, femur, tibia, basitarsus and 2nd tarsal segment of female hind leg; 7, basitarsus and 2nd tarsal segment of male hind leg; 8, median portion of the ventral surface of female abdominal segment 7 showing weakly-sclerotized sternal plates and hair clusters; 9, sternite 8, anterior gonapophyses, genital fork, right paraproct, right cercus (left paraproct and cercus omitted) and spermatheca of female genitalia *in situ* (ventral view); 10, right paraproct and cercus (lateral view); 11, coxites, styles and ventral plate of male genitalia *in situ* (ventral view); 12, right style showing a spiny basal protuberance (end view); 13, ventral plate and median sclerite (lateral view); 14, ventral plate (end view); 15, left paramere and aedeagal membrane (end view); 16, and 17, 10th abdominal segment and cercus (16, lateral view; 17, end view); 18, pupal gill filaments (left side, lateral view); 19, basal portion of pupal gill filaments; 20, apical portion of larval mandible. Scale bars: 0.2 mm for fig. 18; 0.1 mm for figs. 6, 7 and 19; 0.05 mm for fig. 1; 0.03 mm for figs. 2-5, 8-17; 0.02 mm for fig. 20.



Photographs A-E. Cocoon and larva of *S. weji* sp. nov. A, cocoon and pupal exuvia thickly covered with deposits (probably calcium carbonate); B and C, mature larva (B, dorsal view; C, lateral view); D, larval head capsule (dorsal view) showing a negative headspot pattern on cephalic apotome; E, larval head capsule (ventral view) showing a triangular postgenal cleft. Scale bars: 1.0 mm for photos. A, B and C; 0.2 mm for photos. D and E.

surface of 2nd segment mostly dark yellow except narrow portion along posterior margin medium brown, those of other segments brownish black and with dark hairs; segments 2, 5, 6 and 7 each with a pair of silvery iridescent spots dorsolaterally, those on segment 2 connected broadly to each other in middle, those on other segments connected narrowly to each other anteriorly. ***Genitalia*** (Figs. 11-17). Coxite in ventral view nearly quadrate; style elongate, ca. 3.2× as long as its greatest width near middle, ca. 1.6× as long as coxite, curved inward from middle to apex, and with a terminal spine; style with basal protuberance produced dorsomedially, bearing numerous spinules on its surface. Ventral plate with base shorter than wide, rounded laterally, concave medially on posterior margin, having a ventrally-produced process which is setose on anterior and posterior surfaces, and with posterolateral margins dentate though becoming indefinite apically; basal arms of ventral plate diverging from each other. Median sclerite moderately sclerotized, plate-like, widening apically, with rounded apex. Parameres broad basally, each with several hooks apically. Aedeagal membrane moderately setose. Abdominal segment 10 with 6 or 7 short hairs on each posterolateral corner (4 or 5 ventrally and 2 or 3 laterally). Cerci rounded, each with 7-9 short hairs.

Pupa. Body length (excluding gill filaments) 2.0-2.5 mm. ***Head.*** Integument dark yellow to light brown, densely and ellaborately covered with small cone-like tubercles, and with 3 pairs of simple trichomes; 2 frontal trichomes on each side slender and short, 0.4-0.6× as long as facial medium-long trichome, and moderately separated from each other. ***Thorax.*** Integument dark yellow to light brown, densely and ellaborately covered with small cone-like tubercles on anterior 1/2, and moderately or sparsely with smaller cone-like tubercles on posterior 1/2; pit-like cuticular organ absent; thorax with 8 pairs of simple medium-long trichomes (2 anterodorsally, 2 anterolaterally, 1 posterolaterally and 3 ventrolaterally). Gill (Figs. 18 and 19) with 6 thread-like filaments arranged in 3 pairs; upper pair very shortly stalked but middle and lower pairs almost sessile; filaments of dorsal and middle pairs arising side by side, while those of ventral pair vertically; filaments decreasing in length and diameter from dorsal to ventral with outer filament of dorsal pair longest and thickest of all (1.5-1.7 mm long), and lower filament of ventral pair shortest (0.5-0.9 mm long); all filaments pale yellow to medium brown, gradually tapered toward apex, with marked annular ridges and furrows (though ridges becoming indistinct apically), densely covered with minute tubercles. ***Abdomen.*** Tergum 1 with 1 medium-long simple slender hair on each side. Tergum 2 on each side with 1 medium-long simple

hair and 4 short simple spinous setae. Terga 3 and 4 each with 4 hooked spines along posterior margin on each side. Terga 5-7 without spine-combs though tergum 7 with a transverse row of comb-like groups of minute spines; terga 8 and 9 each with a transverse row of distinct spine-combs on each side. Tergum 9 without terminal hooks. Sternum 4 with 1 simple hook and 1 simple seta submedially on each side. Sternum 5 with a pair of bifid hooks submedially on each side. Sterna 6 and 7 each with a pair of bifid inner and simple (or bifid) outer hooks, widely spaced on each side. Grapnel-like hooklets absent. ***Cocoon*** (Photo. A). Simple, slipper-shaped, tightly woven, not extending ventrolaterally; 2.5-3.0 mm long × 0.9-1.1 mm wide.

Mature larva. Body length 4.8-5.0 mm. Body color dark greenish on thoracic segment 1 and abdominal segments 5-8, and light brown or light green on the rest (Photos. B and C), or almost entirely dark greenish. Cephalic apotome (Photo. D) medium brown with pale areas medially and posteriorly. Lateral and ventral surfaces of head capsule dark brown with negative spots (Photo. E). Antenna composed of 3 segments and apical sensillum, much longer than stem of labral fan; length ratio of segments (from base to tip) 1.0:1.0:0.6. Labral fan with ca. 42 main rays. Mandible (Fig. 20) with mandibular serrations composed of 1 medium tooth and 1 small one, without supernumerary serrations; comb-teeth decreasing in length from 1st to 3rd. Hypostomal teeth 9 in number, median tooth and each corner tooth longer than others; lateral margins moderately serrate apically; 5 or 6 hypostomal bristles diverging posteriorly from lateral border on each side. Postgenal cleft (Photo. E) deep, triangular, pointed anteriorly, ca. 3.1× as long as postgenal bridge. Thoracic cuticle almost bare. Abdominal cuticle bare except last segment moderately covered with short colorless setae on each side of anal sclerite. Rectal papilla of 3 lobes, each with 14-16 finger-like secondary lobules. Anal sclerite X-shaped, with broadened base, posterior arms ca. 1.4× as long as anterior ones. Last abdominal segment slightly bulged laterally but lacking ventral papillae. Posterior circlet with ca. 86 rows of hooklets with up to 17 hooklets per row.

TYPE SPECIMENS. Holotype female, slide mounted together with pupal exuvia, Tharn Thong Waterfall, northern Lampang Province, near the border to Lamphun Province, northern Thailand. 15. VI. 2001, by H. Takaoka, W. Choochote, and T. Suntaravitun. Paratypes 5 females, 7 males, 3 pupae, 6 pupal exuviae and 4 mature larvae, same data as holotype.

ECOLOGICAL NOTES. The pupae and larvae of *S. weji* were found to be attached to trailing grasses and stones in a moderately-flowing stream 1.0-3.0 m wide, exposed to the sun. Most of the pupae and their cocoons of this species, as well as stones in the streambed and submerged trailing grasses, were covered with a thick layer of calcareous sediments (Photo. A). Altitude was 580 m. Water temperature was 23℃. No other black-fly species was found in the same stream.

ETYMOLOGY. The species name *weji* is given after Mr. Wej Choochote, Associate professor, Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand, for his great contribution in the research field of Parasitology and Medical Entomology as well as his kind cooperation during the present survey.

REMARKS. This new species is assigned to the *tuberosum* species-group within the subgenus *Simulium* Latreille s. str. by the shape of the genitalia of both sexes and the pupal gill with six filaments per side (Takaoka and Davies, 1996), and seems to be closely related to *S. rufibasis* Brunetti, *S. ramosum* Puri, and *S. fasciatum* Puri, all originally described from India (Brunetti, 1911; Puri, 1932), and also to two unnamed species (*S.* sp. D and *S.* sp. E), reported only from adult female specimens collected together with *S. rufibasis* from Thailand (Takaoka and Suzuki, 1984) by having a pair of hair clusters on the ventral surface of the seventh abdominal segment of the female, which are, though, much longer and fewer in this new species (Fig. 8). The female of *S. weji* is however distinguished from those of all the five known species by the dark hind tibiae leaving only a small pale area basally (Fig. 6) (cf. the hind tibiae of the other species are dark on apical 3/5 to 1/2 and pale on the basal 2/5 to 1/2). The pupa of *S. weji* is easily separated from those of *S. rufibasis* and *S. fasciatum* by the almost sessile gill filaments (cf. gill filaments are stalked in the latter two species), and from *S. ramosum* by the simple trichomes on the head and thorax (cf. trichomes are branched in the latter).

This new species seems to be also related to *S. nigrifacies* Datta from India (Datta, 1974) and *S. puliense* Takaoka from Taiwan (Takaoka, 1979) by having the similar arrangement of the short pupal gill filaments. The female of the latter two species are still unknown. However, the male of these two species are easily separated by the absence of a pair of silvery iridescent spots dorsolaterally on the fifth abdominal segment. In the pupa, there is a slight difference: the tubercles on the head and thoracic integuments are cone-shaped in this new species while those are disc-like in *S. nigrifacies* or round in *S. puliense.*

This new species is distinct from *S. brevipar* and *S.*

tani, both originally described from Peninsular Malaysia (Takaoka and Davies, 1995) and later recorded from Thailand (Takaoka and Saito, 1996), by having the hair clusters on the ventral surface of the seventh abdominal segment in the female, the ventral plate rounded laterally (Fig. 11) in the male, and the cone-shaped tubercles on the head and thoracic integuments in the pupa.

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REFERENCES

1) Brunetti, E. (1911): New Oriental Nematocera. Rec. Indian Mus., 4, 259-316
2) Datta, M. (1974): Some black flies (Diptera: Simuliidae) of the subgenus *Simulium* Latreille (s. str.) from the Darjeeling area (India). Oriental Insects, 8, 15-27
3) Kuvangkadilok, C. and Takaoka, H. (2000): Taxonomic notes on Simuliidae (Diptera) from Thailand: Description of a new species and new distributional records of nine known species. Jpn. J. Trop. Med. Hyg., 28, 167-175
4) Puri, I.M. (1932): Studies on Indian Simuliidae. Part II. Descriptions of males, females and pupae of *Simulium rufibasis* Brunetti, its variety *fasciatum* nov. var. and of three new species from the Himalayas. Indian J. Med. Res., 19, 899-915
5) Takaoka, H. (1979): The black flies of Taiwan (Diptera: Simuliidae). Pacific Insects, 20, 365-403
6) Takaoka, H. and Adler, P.H. (1997): A new subgenus, *Simulium* (*Daviesellum*), and a new species, *S.* (*D.*) *courtneyi* (Diptera: Simuliidae) from Thailand and Peninsular Malaysia. Jpn. J. Trop. Med. Hyg., 25, 17-27
7) Takaoka, H. and Davies, D.M. (1995): The black flies (Diptera: Simuliidae) of West Malaysia. viii + 175p., Kyushu University Press, Fukuoka
8) Takaoka, H. and Davies, D.M. (1996): The black flies (Diptera: Simuliidae) of Java, Indonesia. viii + 81p., Bishop Mus. Bul. Entomol., 6, Bishop Museum Press, Hawaii.
9) Takaoka, H. and Kuvangkadilok, C. (1999): Four new species of black flies (Diptera: Simuliidae) from Thailand. Jpn. J. Trop. Med. Hyg., 27, 497-509
10) Takaoka, H. and Saito, K. (1996): A new species and new records of black flies (Diptera: Simuliidae) from Thailand. Jpn. J. Trop. Med. Hyg., 24, 163-169
11) Takaoka, H. and Suzuki, H. (1984): The blackflies (Diptera: Simuliidae) from Thailand. Jpn. J. Sanit. Zool., 35, 7-45

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MECHANISMS OF PARASITE ANTIGEN UPTAKE FROM THE INTESTINE OF RATS INFECTED WITH THE NEMATODE *NIPPOSTRONGYLUS BRASILIENSIS*

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Abstract: The mechanisms of antigen-uptake at the host mucosal barrier during infection with lumen-dwelling intestinal parasites have not been well elucidated. In this study, we investigated immunolocalization of parasite excretory/secretory (ES) products in the small intestinal epithelium of rats infected with *Nippostrongylus brasiliensis*. Immunoelectronmicroscopy revealed intense ES immunoreactivity in the absorptive cell cytoplasm in association with lysosome-associated membrane glycoprotein (LAMP-1), and in the paracellular spaces. These results indicated that nematode antigen could be absorbed by two pathways: absorption by endocytosis and transport to lysosomes, and absorption through the paracellular pathway, suggesting altered mucosal barrier function.
Key word: *Nippostrongylus brasiliensis*, Rat, Intestine, Parasite antigen, Uptake

INTRODUCTION

Infection of the lumen-dwelling intestinal nematode *Nippostrongylus brasiliensis* induces various immune responses such as IgE antibody production and mastocytosis which are associated with the generation of Th2 cells (Matsuda *et al.*, 1995). Along with immune reactions, *N. brasiliensis* infestation also induces pathological alterations in the jejunal epithelium, such as partial villus atrophy, increases in epithelial cell turnover and decreases in expression of bruch border enzymes (Hyoh *et al.*, 1999; Perdue *et al.*, 1989). Pathophysiological studies have also revealed changes in intestinal permeability after infection: decrease in glucose absorption rate, increased lactulose/mannitol ratio (Nolla *et al.*, 1985; Cobden *et al.*, 1979) and protein leakage into the intestinal lumen which results in hypoalbuminemia (Nawa, 1979; Lunn *et al.*, 1986). Inductions of immunological responses and pathological alterations require absorption of parasite-derived molecules across intestinal epithelium, which normally have a barrier function to prevent absorption of potentially harmful materials from the intestinal lumen: uptake of macromolecules by normal absorptive cells is limited and paracellular channels are sealed by tight junctions (Anderson and Van, 1995). In this context, it is interesting to know the route of absorption of *N. brasiliensis*-derived molecules through the intestinal epithelium of infected animals. In the present study, we examined ultrastructural localization of excretory/secretory (ES) product of *N. brasiliensis* in the rat intestinal epithelium. ES product of *N. brasiliensis* comprise biologically active molecules such as acetylcholinesterase, cysteine proteinase, an interferon-γ suppressive factor and major allergens which evoke marked IgE antibody response in the host (Yamada *et al.*, 1991; Kamata *et al.*, 1995; Nakazawa *et al.*, 1995; Uchikawa *et al.*, 2000).

MATERIALS AND METHODS

Male Spragus-Dawley rats were purchased from SLC Inc. (Shizuoka, Japan). Animals at 8 weeks of age were infected with 2,000 *N. brasiliensis* L3 larvae by subcutaneous injection. ES product form adult *N. brasiliensis* worms were prepared as described (Yamada *et al.*, 1991). Polyclonal antibody against ES product was raised in Japanese white rabbits by injecting 10 μg ES with Freund's complete adjuvant 3 times every 4 weeks, and the IgG fraction was prepared using a protein-G column (Amersham Pharmacia Biotech, Buckinghamshire, UK). Immunoblotting analyses showed that this antibody recognized multiple molecules of ES product from 17-220 kDa, but not of normal intestinal

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lysates of mice and rats (data not shown).

Preliminary immunohistochemical studies using paraffin-embedded tissue sections of the proximal jejunum showed that there was intense ES immunoreactivity in the villus epithelium 7 and 10 days p.i., while not in uninfected animals or those 3, 14 and 21 days p.i., suggesting that absorption of ES product is detectable during the period when a large numbers of adult worms are parasitized in the lumen. Therefore, immunoelectronmicroscopic studies were carried out 7 days p.i. using 4 rats.

For immunoelectron microscopy, segments of the jejunum approximately 20 cm distal to the pyloric ring were removed and fixed in a solution containing 0.5% glutaraldehyde, 4% paraformaldehyde and 0.1 M phosphate buffer, pH 7.4, for 1 hr at 4℃, and embedded in LR White resin (London Resin Co., Surrey, UK) in a position so that villus structures were cut longitudinally. Ultra-thin sections were cut and incubated with 5% (w/v) non-fat dry milk, followed by incubation with anti-ES rabbit IgG (50 μg/m*l*) overnight at 4℃. For double immunostaining of ES and lysosome associated membrane glycoprotein-1 (LAMP-1), sections were further incubated with anti-LAMP-1 goat IgG (2 μg/m*l*: Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 hr at room temperature. After washing, sections were incubated with 15 nm gold particles-conjugated goat anti-rabbit IgG (diluted to 1:20) or 5 nm gold particles-conjugated rabbit anti-goat IgG (diluted to 1:20) (BB International, Golden gate, UK) for detections of ES and LAMP-1 for 1 hr at room temperature, respectively, followed by staining with 2% uranyl acetate, and observed under a Hitachi H-7000 electron microscope.

RESULTS AND DISCUSSION

In uninfected animals, ES-specific antibody showed negligible levels of non-specific binding to absorptive cell cytoplasm, and moderate levels of nonspecific binding to microvilli (Fig. 1a). Seven days p.i., intense immunoreactivity was found on microvilli, paracellular spaces and the cytoplasm of villus absorptive cells, in which some immunoreactivity was localized to vesicular structures (Fig. 1b). Low level ES immunoreactivity was also found on the cellular membrane along the basal lamina (Fig. 1c). In a few absorptive cells, there was markedly dense immunoreactivity in the lysosome-like organelle (Fig. 1d). To determine whether ES product is transported to absorptive cell lysosomes, double immunostaining of ES and LAMP-1 was carried out. As shown in Fig. 2a, b, at least some of ES immunoreactivity showed co-localization with LAMP-1. These localizations of immunoreactivity were not observed in all villus absorptive cells, but in patchy distributions with frequencies higher in the upper part of villi. Crypt lining cells did not reveal ES immunoreactivity, while M cells over Peyer's patches showed similar distribution of ES immunoreactivity to that of villus absorptive cells (data not shown). Using nonimmune rabbit or goat IgG, there was no gold particle deposition in paracellular spaces or in the cytoplasm of absorptive cells.

It has been presumed that only M cells over Peyer's patches are specialized for the uptake of macromolecules and antigen presentation to T cells (Mayer, 1997). However, the present studies showed that parasite-derived molecules could be transported not only through paracellular spaces but also absorbed in the villus epithelial cells, indicating that the normal barrier functions of intestinal epithelium are disrupted after infection of lumen-dwelling nematodes. The mechanism of the antigen uptake through paracellular channels is not clear from the present study. It is possible that nematode infection altered expression of adhesion molecules of villus epithelial cells. In fact, we reported previously that expression of E-cadherin, a major epithelial cell adhesion molecule, was significantly reduced at the tip of villi after *N. brasiliensis* infection (Hyoh *et al.*, 1999). Recently, it was reported that a cysteine proteinase allergen Der pl is transported through the paracellular pathway of respiratory epithelium possibly mediated by the breakdown of tight junctions by the allergen's own proteolytic activity (Wan *et al.*, 1999). In this respect, it is interesting that *N. brasiliensis* ES product also contains a cysteine protease (Kamata *et al.*, 1995). Alternatively, increase of paracellular permeability might have been triggered indirectly by signals from absorptive cells, as suggested by reports that the solute permeation across tight junctions is not static and can be reversibly regulated by intracellular events with a common effector mechanism probably tied to the cytoskeleton (Madara, 1998).

The present results also showed that the ES product was absorbed in the villus epithelial cells, and at least some ES immunoreactivity was co-localized with LAMP-1, suggesting that ES product was internalized by endocytosis and transported into lysosomes. These results are consistent with the report that at least some species of macromolecules can be transported across the normal mature gut by a pinocytotic processes (Walker and Isselbacher, 1974). Since villus epithelial cells express MHC class II antigens and its expression was enhanced after *N. brasiliensis* infection (Mayrhofer and Spargo, 1990; Masson and Perdue, 1990), it is possible that some of the processed antigens are directly presented from intestinal absorptive cells to lymphocytes.

Taken together, the present studies showed that *N. brasiliensis* antigen is partly internalized in intestinal ab-

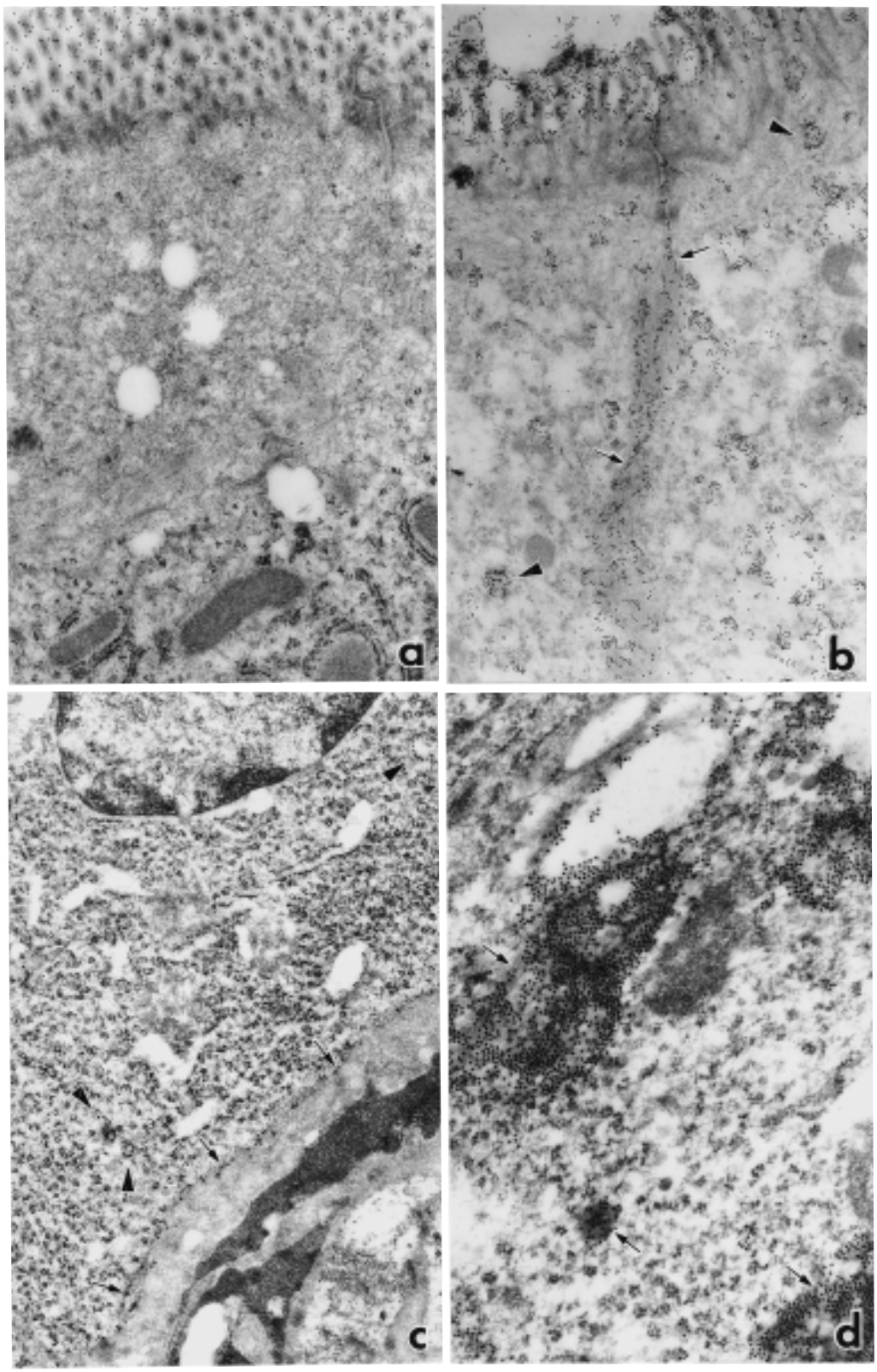

Figure 1 ES immunoelectronmicroscopy of rat small intestinal villus epithelium from uninfected rats (a) or animals 7 days after *N. brasiliensis* infection (b-d). *a*: nonspecific gold-particle deposition is noted on microvilli, while deposition in the absorptive cell cytoplasm and paracellular spaces are negligible. ×20,000. *b*: Intense ES immunoreactivity is noted on microvilli, paracellular spaces (arrows), and in the cytoplasmic vesicular structures (arrowheads). ×20,000. *c*: ES immunoreactivity is observed on the basal cell membrane (arrows) and in a small vesicular structures (arrowheads) of absorptive cells. ×21,360. *d*: Intense immunoreactivity is noted in lysosome-like structures (arrows). ×34,860.

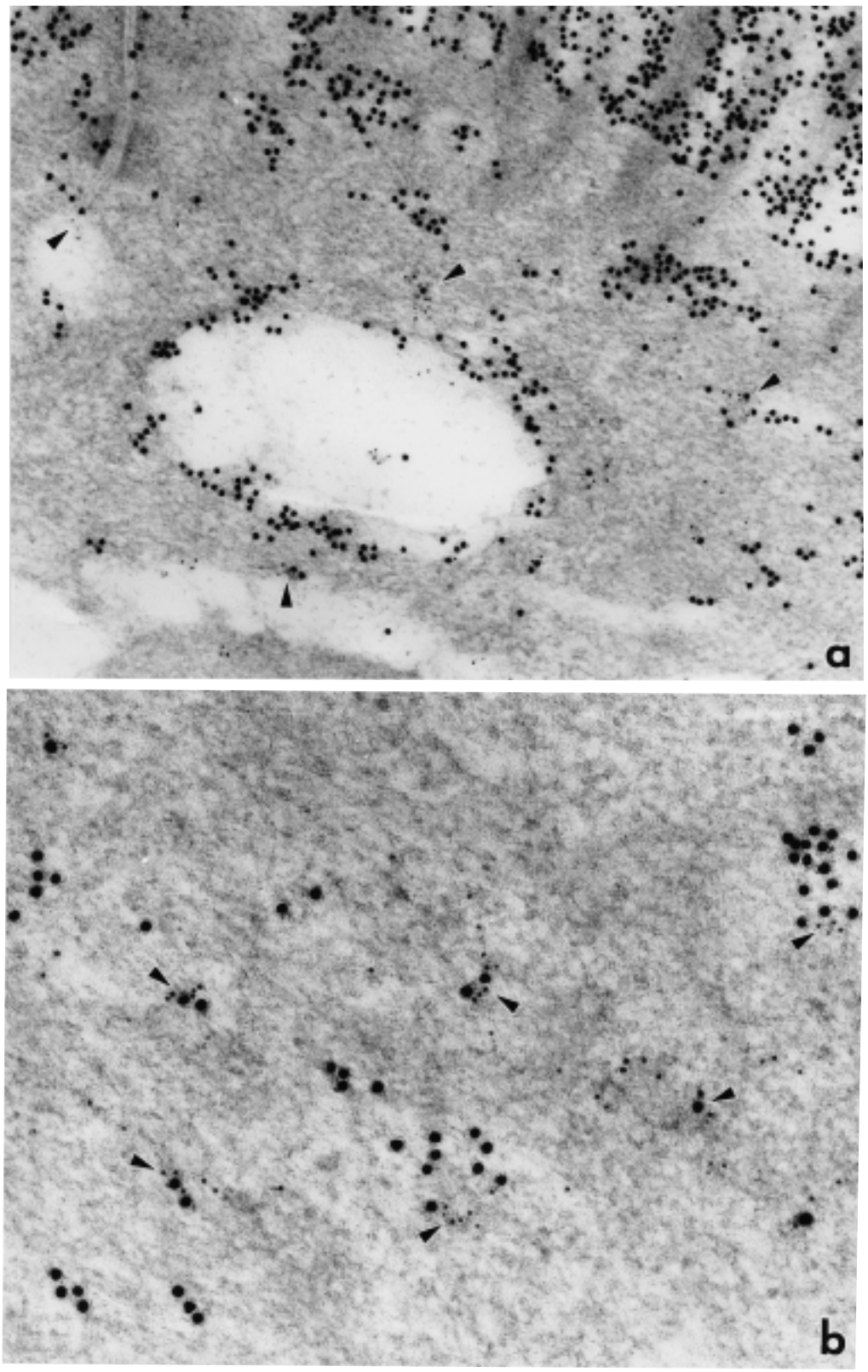

Figure 2 ES (large gold particles) and LAMP-1 (small gold particles) double immunostaining of rat small intestinal villus epithelium from rats 7 days after *N. brasiliensis* infection. ES and LAMP-1 (arrowheads) are occasionally co-localized in membrane-bound structures in the cytoplasm of absorptive cells. *a*: ×72,000. *b*: × 134,000.

sorptive cells and partly transported through the paracellular pathway, probably in association with the disruption of barrier function. The uptake of parasite antigen through the intestinal epithelium would have primary importance for the induction of immune reactions and development of intestinal pathology.

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REFERENCES

1) Anderson, J.M. and Van, I.C. (1995): Tight junctions and the molecular basis for regulation of paracellular permeability. Am. J. Physiol., 269, g467-475
2) Cobden, I., Rothwell, J. and Axon, A.T. (1979): Intestinal permeability in rats infected by *Nippostrongylus brasiliensis.* Gut, 20, 716-721
3) Hyoh, Y., Nishida, M., Tegoshi, T., Yamada, M., Uchikawa, R., Matsuda, S. and Arizono, N. (1999): Enhancement of apoptosis with loss of cellular adherence in the villus epithelium of the small intestine after infection with the nematode *Nippostrongylus brasiliensis* in rats. Parasitology, 119, 199-207
4) Kamata, I., Yamada, M., Uchikawa, R., Matsuda, S. and Arizono, N. (1995): Cysteine protease of the nematode *Nippostrongylus brasiliensis* preferentially evokes an IgE/IgG1 antibody response in rats. Clin. Exp. Immunol., 102, 71-77
5) Lunn, P.G., Northrop, C.A., Behrens, R.H., Martin, J. and Wainwright, M. (1986): Protein losing enteropathy associated with *Nippostrongylus brasiliensis* infestation and its impact on albumin homoeostasis in rats fed two levels of dietary protein. Clin. Sci., 70, 469-475
6) Madara, J.L. (1998): Regulation of the movement of solutes across tight junctions. Ann. Rev. Physiol., 60, 143-159
7) Masson, S.D. and Perdue, M.H. (1990): Changes in distribution of Ia antigen on epithelium of the jejunum and ileum in rats infected with *Nippostrongylus brasiliensis.* Clin Immunol. Immunopathol., 57, 83-95
8) Matsuda, S., Uchikawa, R., Yamada, M. and Arizono, N. (1995): Cytokine mRNA expression profiles in rats infected with the intestinal nematode *Nippostrongylus brasiliensis.* Infect. Immun., 63, 4653-4660
9) Mayer, L. (1997): Local and systemic regulation of mucosal immunity. Aliment. Pharmacol. Ther., 3, 81-85
10) Mayrhofer, G. and Spargo, L.D. (1990): Distribution of class II major histocompatibility antigens in enterocytes of the rat jejunum and their association with organelles of the endocytic pathway. Immunology, 70, 11-19
11) Nakazawa, M., Yamada, M., Uchikawa, R. and Arizono, N. (1995): Immunocytochemical localization of secretory acetylcholinesterase of the parasitic nematode *Nippostrongylus brasiliensis.* Cell. Tissus Res., 280, 59-64
12) Nawa, Y. (1979): Increased permeability of gut mucosa in rats infected with *Nippostrongylus brasiliensis.* Int. J. Parasitol., 9, 251-255
13) Nolla, H., Bristol, J.R. and Mayberry, L.F. (1985): *Nippostrongylus brasiliensis*: malabsorption in experimentally infected rats. Exp. Parasitol., 59, 180-184
14) Perdue, M.H., Ramage, J.K., Burget, D., Marshall, J. and Masson, S. (1989): Intestinal mucosal injury is associated with mast cell activation and leukotriene generation during *Nippostrongylus*-induced inflammation in the rat. Dig. Dis. Sci., 34, 724-731
15) Uchikawa, R., Matsuda, S. and Arizono, N. (2000): Suppression of gamma interferon transcription and production by nematode excretory-secretory antigen during polyclonal stimulation of rat lymph node T cell. Infect. Immun., 68, 6233-6239
16) Walker, W.A. and Isselbacher, K.J. (1974): Uptake and transport of macromolecules by the intestine. Possible role in clinical disorders. Gastroenterology, 67, 531-550
17) Wan, H., Winton, H.L., Soeller, C., Tovey, E.R., Gruenert, D.C., Thompson, P.J., Stewart, G.A., Taylor, G.W., Garrod, D.R., Cannell, M.B. and Robinson, C. (1999): Der pl facilitates transepithelial allergen delivery by disruption of tight junctions. J. Clin. Invest., 104, 123-133
18) Yamada, M., Nakazawa, M., Matsumoto, Y. and Arizono, N. (1991): IgE antibody production in rats against multiple components of excretory-secretory products of the nematode *Nippostrongylus brasiliensis.* Immunology, 72, 104-108

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INDUCTION OF IGE ANTIBODY RESPONSE IN MICE BY CONTINUOUS ADMINISTRATION OF EXCRETORY/SECRETORY PRODUCTS OF THE NEMATODE *NIPPOSTRONGYLUS BRASILIENSIS*

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Abstract: IgE-inducing potential of excretory/secretory products (ES) of the nematode *Nippostrongylus brasiliensis* was examined in mice immunized with ES without use of adjuvant. Repeated i.p. injections or continuous i.p. or s.c. infusion of ES induced significant increases in total serum IgE. Continuous i.p. or s.c. infusion of ES also induced ES-specific IgE antibody, while repeated i.p. injections did not. IL-4 production was increased by ex vivo stimulation with ES of mesenteric lymph node cells from repeatedly immunized mice. On the other hand, continuous intraduodenal infusion of ES induced neither total nor specific IgE responses. These results suggest that continuously secreted ES from the nematode are relevant for the up-regulation of IL-4 and IgE responses, although additional factor(s) may be necessary for the uptake of ES across the intestinal mucosal barrier.
Key words: *Nippostrongylus brasiliensis*, IgE, Mice, Continuous administration, Excretory/secretory products

INTRODUCTION

Previously, we showed that excretory/secretory products (ES) of *N. brasiliensis* contain several major antigens of 16 to 130 kDa including some functional molecules such as acetylcholinesterase as well as cysteine protease, that are produced in and secreted from the subventral gland of the worm (Burt and Ogilvie, 1975; Kamata *et al.,* 1995; Nakazawa *et al.,* 1995; Yamada *et al.,* 1991; Yamada *et al.,* 1993). Intraperitoneal administration of a single large dose of ES induced a small but significant increase in the level of total serum IgE, but not ES-specific IgE in naive rats (Uchikawa *et al.,* 1993). The aim of the present study was to examine whether repeated or continuous ES administration without use of adjuvant evokes IgE antibody production in high-IgE responder BALB/c mice.

MATERIALS AND METHODS

Male BALB/c mice, 8 weeks old, were purchased from Japan SLC Inc. (Hamamatsu, Japan). The strain of *N. brasiliensis* used has been maintained in our laboratory by serial passage in rats. ES was prepared from *N. brasiliensis* as described previously (Yamada *et al.,* 1991).

In one experiment, ES was dissolved in PBS (10 or 100 μg/m*l*) and was injected i.p. at various time intervals. A group of mice was inoculated i.p. with adult *N. brasiliensis* worms which had been collected from the small intestine of a rat 7 days p.i. In another experiment, a total dose of 320 μg ES was continuously delivered by mini-osmotic pumps (Alzet model 2001, Alza Corp., Palo Alto, CA) at the rate of 1 μ*l*/hr for 7 days. For i.p. administration, mini-osmotic pumps were implanted into the peritoneal cavity and for s.c. administration into the dorsal flank. For intraduodenal administration, mini-pumps were implanted subcutaneously into the dorsal flank tissue and a draining catheter attached to the pump was passed through the subcutaneous tissue and introduced into the peritoneal cavity. The tip of the catheter was inserted into the duodenal lumen through a small incision and the catheter was secured to the duodenal wall with Histoacrylblue (B.Braun Melsüngen AG, Melsüngen, Germany). All skin incisions were closed with suture silk.

For measurements of total serum IgE, IgG1 or IgG2a by ELISA, the wells of 96-well plates were coated with rat mAb against mouse IgE (10 μg/m*l*: LO-ME-2; EIU Inc., Belgium), IgG1 (2 μg/m*l*: LO-MG1-2, Zymed Laboratories Inc., San Francisco, USA), or IgG2a (2 μg/m*l*: LO-MG2a-

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3; Zymed Laboratories Inc.) dissolved in carbonate buffer, pH 9.6, overnight at 4℃. After washing, the wells were incubated with bovine milk protein (Block Ace; Dainihon Pharm. Co., Osaka, Japan), followed by incubation with serially diluted test sera for 2hr. The plates were then incubated with goat anti-mouse IgE serum (Bethyl Laboratories Inc., Montgomery, USA), followed by incubation with affinity-purified biotin-conjugated rabbit IgG against goat IgG (MBL Inc., Nagoya, Japan) or for detection of IgG1 or IgG2a, the plates were incubated with biotin-conjugated anti-mouse IgG1 or IgG2a rat monoclonal antibodies (Zymed Laboratories Inc.). After subsequent incubation with alka-line phosphatase-labeled streptavidin (Boehringer Mannheim Biochemica, Mannheim, Germany), 10 mM 4-nitrophenylphosphate dissolved in 10 mM diethanolamine buffer (pH 9.8) containing 0.5 mM $MgCl_2$ was added as a substrate. The OD value at 405 nm was measured with a microplate reader. Standard curves were generated by serial dilutions of purified IgE protein (mouse anti-DNP IgE monoclonal antibody; Yamasa Shoyu Co., Tokyo, Japan), mouse IgG1 myeloma protein, or mouse IgG2a myeloma protein (Zymed Laboratories Inc.) and amounts of IgE, IgG1 and IgG2a in test sera are expressed in μg/m*l*.

For detection of ES-specific IgE, IgG1 or IgG2a by ELISA, the wells were coated with ES (10 μg/m*l*) dissolved in carbonate buffer, pH 9.6, overnight at 4℃. ES-coated plates were further incubated with bovine milk protein, then with test sera (1:10 dilution) for 2 hr. The plates were further incubated with biotin-conjugated rat mAb against mouse IgE (PharMingen, San Diego, USA), IgG1 (Zymed Laboratories Inc.) or IgG2a (PharMingen) followed by incubation with horseradish peroxidase-labeled streptavidin (Amersham, Bucks, U.K.). Reaction with o-phenylenediamine was carried out, and the OD value at 492 nm was determined.

For measurement of IL-4 production, mesenteric lymph nodes (MLN) were removed from mice, and single cells were prepared and cultured with ES as described previously (Tegoshi *et al.,* 1997). Concentrations of IL-4 in culture supernatant were determined using ELISA kits (Endogen Inc., Cambridge, USA) according to the manufacturer's instructions.

Statistical analysis was performed using Mann-Whitney *U*-test.

RESULTS AND DISCUSSION

To determine whether antigens released from adult worms have the potential to induce IgE antibody responses, living adult worms were transplanted into the peritoneal cavity of naive mice. These mice developed significant increases of total and specific IgE levels (Table 1, Group F). Most worms had penetrated mainly to the omentum and peripancreatic tissue, by day 7. They were still alive on day 7, and a few had survived by day 14 (Arizono *et al.,* 1994). It was assumed that these transplanted worms potentiated IgE production. When mice received repeated i.p. injections of 10 μg ES (Table 1, Group B, C), no IgE response was observed, while administration of 100 μg of ES 5 times every 3 days induced significant increases in total serum IgE (Table 1, Group E). Although specific IgE was not detectable in these mice, ex vivo stimulation of MLN cells with ES induced significant IL-4 response (56.3±23.5 pg/m*l* in mice administered with 100 μg ES 5 times every 3 days i.p. vs <5 pg/m*l* in mice injected with PBS 5 times every 3 days i.p.).

Table 1 IgE antibody responses after repeated ES administration or transplantation of adult *N. brasiliensis* worms into the peritoneal cavity of mice

Group	Treatment	Total quantity of ES (μg)	Total IgE (μg/m*l*)	ES-specific IgE (O.D.)
A	PBS	-	0.12±0.04	0.094±0.023
B	10 μg ES	20	0.25±0.13	0.080±0.031
C	10 μg ES	50	0.52±0.50	0.079±0.023
D	100 μg ES	200	0.23±0.07	0.059±0.009
E	100 μg ES	500	2.53±1.31**	0.079±0.017
F	Adult worm	-	1.79±0.61*	0.300±0.120*

A: PBS was injected i.p. five times every three days. B, C: 10 μg of ES was injected i.p. twice at 1 week interval (B), or five times every three days (C). D, E: 100 μg of ES was injected i.p. twice at 1 week interval (D), or five times every three days (E). F: Adult worms (500) were surgically transplanted into the peritoneal cavity. Sera were obtained 14 days after the first injection of ES or surgical transplantation of adult worms. Data shown are means±S.D. of 4-6 mice. Significantly different from the values for group A: *p<0.05, **p<0.01 (Mann-Whitney *U*-test)

Next, we examined IgE responses in mice after continuous infusion of ES in the peritoneal cavity, subcutaneous tissue or duodenum using implanted mini-osmotic pumps. Two weeks after implantation of the pumps, i.p. and s.c. routes of administration induced significant increases in the levels of total and specific IgE (Table 2). Total and specific IgG1 levels were also increased significantly following i.p. and s.c. administration (Figure 1A, B). On the other hand, IgG2a levels did not change significantly (data not shown). With intraduodenal administration, neither IgE (Table 2), nor IgG1 (Figure 1A, B) or IgG2a (data not shown) responses developed.

The reason for the failure of specific IgE response by repeated i.p. administration of ES, despite ES-specific IgE response developed by continuous i.p. or s.c. administration, is not clear. It is possible that repeated exposure of ES antigen with short intervals might rather suppressed the specific

IgE production.

Previously, we showed that a single i.p injection of large dose of ES to rats induced a low level increase in total serum IgE without production of specific IgE antibodies (Uchikawa *at al.,* 1993). The present results in mice showed that although IgE response did not occur with 10 μg of ES every three days or with 100 μg of ES twice at 1 week interval, administration of 100 μg of ES every three days induced significant increases in total serum IgE. Further, continuous i.p. or s.c. infusion of ES induced significant increases in both total and ES-specific IgE comparable to those observed following implantation of living adult worms into the peritoneal cavity. These results suggest that the critical amount of ES for induction of the IgE response must be over 320 μg, whereas less than 200 μg will not be effective. We previously reported that ES is produced at levels of >200 μg by 1,000 adult *N. brasiliensis* worms per day *in vitro* (Yamada *et al*., 1991). These results are consistent with the observation that levels of total serum IgE after *N. brasiliensis* infection in rats were positively correlated with the numbers of infecting worms, although the nematode-specific IgE antibody response was rather suppressed at very high worm burden (Yamada *et al.,* 1992).

Table 2 IgE antibody responses after continuous ES administration into the duodenal lumen, peritoneal cavity or subcutaneous tissue of mice

	Total IgE (μg/m*l*)	ES-specific IgE (O.D.)
PBS	0.13±0.02	0.020±0.006
intraduodenal	0.45±0.44	0.034±0.012
intraperitoneal	1.30±0.41*	0.199±0.054*
subcutaneous	1.10±0.29*	0.132±0.063*

A total of 320 μg ES was administered by implanted mini-osmotic pumps at a rate of 1 μl/hr for 7 days. Control animals were administered PBS i.p. by mini-osmotic pumps. Sera were obtained 14 days after implantation of the mini-osmotic pumps. Data shown are means ±S.D. of 4 mice. Significantly different from the values for PBS control: $^{*}p<0.05$ (Mann-Whitney *U*-test)

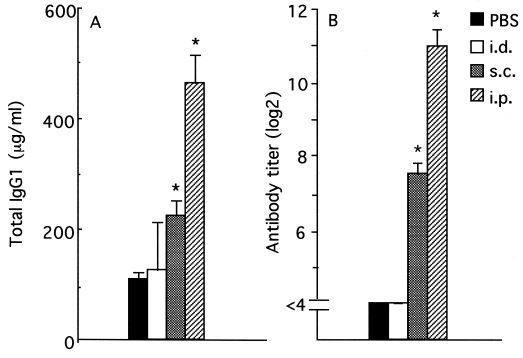

Figure 1 Serum levels of total IgG1 (A) and ES-specific IgG1 (B) antibody 2 weeks after continuous administration of a total of 320 μg ES into the duodenum (i.d.), subcutaneous tissue (s.c.), or peritoneal cavity (i.p.) by Alzet mini-osmotic pumps at the rate of 1 μl/hr for 7 days. Control animals were infused with PBS intraperitoneally for 7 days. The bar on the top of each column represents SE. Significantly different from the values for PBS control: $^{*}p<0.05$.

Taken together, the present results show that ES continuously secreted from nematodes is responsible for triggering IgE antibody responses.

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REFERENCES

1) Arizono, N., Yamada, M., Tegoshi, T., Okada, M., Uchikawa, R. and Matsuda, S. (1994): Mucosal mast cell proliferation following normal and heterotopic infections of the nematode *Nippostrongylus brasiliensis* in rats. APMIS, 102, 589-596
2) Burt, J.S. and Ogilvie, B.M. (1975): *In vitro* maintenance of nematode parasites assayed by acetylcholinesterase and allergen secretion. Exp. Parasitol., 38, 75-82
3) Kamata, I., Yamada, M., Uchikawa, R., Matsuda, S. and Arizono, N. (1995): Cysteine protease of the nematode *Nippostrongylus brasiliensis* preferentially evokes an IgE/IgG1 antibody response in rats. Cli. Exp. Immunol., 102, 71-77
4) Nakazawa, M., Yamada, M., Uchikawa, R. and Arizono, N. (1995): Immunocytochemical localization of secretory acetylcholinesterase of the parasitic nematode *Nippostrongylus brasiliensis.* Cell and Tissue Res., 280, 59-64
5) Tegoshi, T., Okada, M., Nishida, M. and Arizono, N. (1997): Early increase of gut intraepithelial mast cell precursors following *Strongyloides venezuelensis* infection in mice. Parasitology, 114, 181-187
6) Uchikawa, R,, Yamada, M., Matsuda, S. and Arizono, N. (1993): IgE antibody responses induced by transplantation of the nematode *Nippostrongylus brasiliensis* in rats: a possible role of nematode excretory-secretory product in IgE production. Immunology, 80, 541-545
7) Yamada, M., Nakazawa, M., Matsumoto, Y. and Arizono,

N. (1991): IgE antibody production in rats against multiple components of excretory-secretory products of the nematode *Nippostrongylus brasiliensis.* Immunology, 72, 104-108

8) Yamada, M., Nakazawa, M., Kamata, I. and Arizono, N. (1992): Low-level infection with the nematode *Nippostrongylus brasiliensis* induces significant and sustained specific and non-specific IgE antibody responses in rats. Immunology, 75, 36-40

9) Yamada, M., Nakazawa, M. and Arizono, N. (1993): IgE and IgG2a antibody responses are induced by different antigen groups of the nematode *Nippostrongylus brasiliensis* in rats. Immunology, 78, 298-302

EVALUATION OF ANAEROPACK® (AnP) TYPE AS TOOLS OF *PLASMODIUM FALCIPARUM* CULTIVATION AND DRUG SENSITIVITY TESTS

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Abstract: The AnaeroPack (AnP) system (Mitsui Gas Co., Japan) was produced for the purpose of culturing anaerobic bacteria. The idea using AnP Campylo® for cultivation of *Plasmodium falciparum* and its successful results were firstly reported by Haruki *et al.* (1997). In the present study, characterisation of 3 products of AnPs, *i.e.*, AnP CO_2® (at a gas phase of 5% CO_2 and 15% O_2), AnP Campylo® (10% CO_2 and 10% O_2) and AnP Plas® (5% CO_2 and 5% O_2), in an AnaeroPack Box Jar® (22×16×10.5 cm) was studied comparing with conditions in a gas incubator or gas chamber (4% CO_2 and 3% O_2) and in a candle jar (5% CO_2 and 15% O_2). The growth curves of malaria parasites showed no difference among these four conditions by incubation for 48 hr except for AnP Plas. The observed IC_{50} levels of chloroquine were varied much by the difference of AnPs, gas phases, the pressure in a Box Jar and free space inside. For stabilisation of IC_{50} testing, free space in a Box Jar should be kept equal and minimal by placing the maximal 5 test plates by adding dummy plates. By the stable results of IC_{50}, gas phases can be divided into two groups. The one involves the gas incubator, AnP Plas® and AnP Campylo®, and the other does the candle jar and AnP CO_2®. Results of chloroquine sensitivity test in the candle jar group showed more drug sensitive than in the gas incubator group. These two groups should be used properly by the purpose of field experiments. The AnP® system is proven to be a practical tool for malaria culture in the field for occupying only a small space in an incubator.

Key words: Malaria, *Plasmodium falciparum*, *in vitro* culture, AnaeroPack®, Microanaerophilic condition, Chloroquine sensitivity

INTRODUCTION

Since discovery of cultivation method of *P. falciparum* by Trager and Jensen (1976), it has been possible to survey the drug sensitivity using a microtest method which was recommended by Rieckmann *et al.* (1978) and WHO (Bruce-Chwatt *et al.*, 1986). On the other hand, the problem of drug resistance becomes a big obstacle for prevention and treatment of falciparum malaria world widely. It is important to survey the drug sensitivity in malarious areas and to document the extent and distribution of resistance in the world. Therefore, the discrepancy among drug sensitivity assay methods is not only scientific issues but also clinical and practical problems. In addition, the assay system is required to be less laborious and of accuracy, because these tests will be performed usually under unsatisfied facilities and low economical conditions in malaria infested areas.

To produce a stable gas phase in the field studies as in the gas incubator that is approriate for malaria cultivation and drug sensitivity test, an idea of using AnaeroPack (AnP) Campylo® for cultivation of *P. falciparum* was firstly reported by Haruki *et al.* (1997a) at the 9th Malaria meeting of British Society of Parasitology, Liverpool held in 1997, and next in Japan by Haruki *et al.* (1997b) in terms of growth efficiency and drug sensitivity test of *P. falciparum* in comparison with ordinary systems in the gas incubator and in a candle jar. Following the above reports, a few works have been performed using AnP system (Taylor-Robinson, 1998; Onda *et al.*, 1999; Lin *et al.*, 1999; Mizuno *et al.*, 2000). AnP systems is simpler and easier to handle than the technique with a candle jar. In the present study, the other two products, AnP CO_2® and AnP Plas® were added to AnP Campylo®, and their characterisation was studied to clarify how and what purpose individual AnP types should be used properly. The benefit of using AnP system in the AnP Box Jar® was also studied from parasi-

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tological points of view in comparison with systems in a gas incubator and in a candle jar.

MATERIALS AND METHODS

1) Malaria parasites and cultivation procedures

P. falciparum K1, (chloroquire resistant strain) originating from Thailand and HB3, (chloroquine sensitive strain) originating from Hondulus were used (supplied in courtesy of Dr. S.A., Ward of University of Liverpool) for the growth study. K1 is regarded as very useful strain because it shows rather highly different values of IC_{50} than other sensitive strains where it was used for chloroquine sensitivity assays in different gas conditions. After retrieval from cryopreservation, the parasites were maintained according to a modifird method described by Trager and Jensen (1976). RPMI 1640 (Sigma) was used for parasite growth supplemented with 2 mg L-Glutamine, 1.0 g $NaHCO_3$, 12.5 m*l* of 1M HEPES (Dojindo), 0.02 mg gentamycin (Sigma) in 500 m*l*, and 50 m*l* of human AB serum were added (Western Tokyo Red Cross Blood Centre, Tachikawa, Tokyo). A haematocrit of 5% was maintained with washed human blood (type O Rh+) cells supplied also from Tokyo Red Cross Blood Centre. Before incubation at 37℃, the completed culture was gassed using a special gas mixture (Setagaya Sanso) composed of 3% O_2 4% CO_2 and 93% N_2. Cultures were maintained on a daily basis by observing blood films to assess parasitaemia and replenishing the medium to feed. Parasite culture were subcultured regularly usually after parasitaemia reached 5%.

2) Parasite Growth

Parasite growth was observed by culturing in 96 well plate (Falcon 3072) in different gas compositions, 1) Gas chamber flown with special gas mixture containing 3% O_2, 4% CO_2 and 93% N_2 (Setagaya Sanso), 2) AnP Plas® (5% CO_2 and 5% O_2), 3) AnP Campylo® (10% CO_2 and 10% O_2), 4) AnP CO_2® (15% O_2 and 5% CO_2), in an AnP Box-type Jar® (the size: 22×16×10.5 cm, weight: 500 g, Fig 1.) and 5) Candle Jar (15% O_2 and 5% CO_2). Infected erythrocytes were diluted to 1% parasitaemia with fresh red blood cells. Samples were then diluted with RPMI medium in 5% haematcrit, 100 μl each were transferred into wells of six rows and ten column of the plates. The plates were placed in different gas conditions and incubated at 37℃. Every 24 hr, the samples were taken from six wells, fixed with methanol, then stained with 10% Giemsa for 30 min to count parasitaemia with a light microscope. The culture media were changed when the samples were taken. New sachets were placed in the jars to maintain gas condition after sampling. Growth curves were also observed by placing the varied number of the plates in a jar.

Figure 1 AneroPack sachets (from right to left: AneroPack Campylo®, CO_2 and Plas®) and specific jar (AneroPack Kakugata jar®) with five plates.

3) Chloroquine (CQ) sensitivity assay

CQ (Sigma) in 50% methanol (1 mM) was diluted with RPMI medium in serial two hold dilutions at concentrations of 0, 15.6, 31.3, 62.5, 125, 250, 500, 1,000, 2,000 and 4,000 nM. Fifty (50) μl of RPMI containing CQ were placed into wells. Infected erythrocytes diluted into 1% parasitaemia with fresh red blood cells were diluted with RPMI into 10% haematocrit, and 50 μl of sample were added into each well to adjust the final haematcrit at 5% and final concentrations of CQ at 0 (×2), 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1,000 and 2,000 nM. The plates were incubated for 36 hr under different conditions as described above. Culture samples were taken from the wells for Giemsa stain to count the number mature and immature schizonts in infected cells. To determine Percent maturation, the number of schizonts was counted and divided by the number of schizonts in control. The date were analysed using Grafit® programme (Enrithacus Software, Kent, UK) to determine the 50% inhibition concentration (IC_{50}).

4) Gas concentration measurement

Gas concentrations in a jar were measured using Oxygen Analyser LC-700 (Toray Engineering Co., Ltd. Japan) for O_2 and Infrared Gas Analyser UR-025S (Komyo Rikagaku Kogyo KK, Japan) for CO_2 30 min, 1, 2, 4, 18, 24, 48, 72, 96, 120 and 144 hr after start of incubation.

5) Gas pressure measurement in a jar

The pressure in the Box Jar was measured by placing different number of plates in it. The barometer (Rhythm Altimeter, Japan) was read 30, 60, 120 min and 24 hr after

incubation.

RESULTS

Difference of growth rates of *P. falciparum* K1 strain was examined among different gas phases such as in a gas incubator, by 3 products of AnP and in a candle jar. The cultivation started at 1% parasitaemia, and development of parasites was followed by percent parasitaemia after 24 and 48 hr. The result of growth rates thus observed is shown in Table 1, but there was no remarkable difference of growth rates in different AnPs except for AnP plas (Table 1). AnP plas shows less growth with single plate (p=0.003) but not with five plates.

As it was noticed that the growth rates showed some difference by the different number of culture plates placed in a Box Jar, growth rates were suspected to be affected by the free space inside of the Jar, since more time would be required until gas composition becomes stable in the Jar, if the free space is wider. To examine this, the growth rates were compared by placing 1 or 5 plates in a Jar at different gas phases by 2 types of AnP with control plates in a gas incubator and in a candle jar. Using AnP Plas, growth rates were affected by different free space in a Box Jar. The culture in a Jar occupied by five plates shows better growth than by a single plate with AnP Plas (p=0.99) (Table 2).

The results indicated narrower free space in a Jar provided better growth and stable results, therefore, for stabilisation of cultivation in a Jar, 5 plates should be always placed by adding dummy plates besides examining plates up to a total of 5.

Difference of concentrations of CO_2 and O_2 in a Box Jar was measured by placing 1 or 5 plates in a Jar after 24 hr incubation (Table 3).

Of course, there is no difference of growth by the different number of plates in the gas incubator or in a big can-

Table 1 Growth of *Plasmodium falciparum* K1 strain expressed by % parasitaemia at different gas conditions placing 1 culture plate in an AnaeroPack box jar and starting cultivation at 1% parasitaemia

Hours for cultivation jar	Gas	Plas	Campylo	CO_2	Candle
0	1.0±0.2%	1.0±0.2%	1.0±0.2%	1.0±0.2%	1.0±0.2%
24	1.2±0.2%	1.2±0.4%	1.2±0.5%	1.2±0.3%	1.3±0.4%
48	5.3±0.2%	4.2±0.3%	4.8±0.8%	4.5±0.5%	4.5±0.6%

Gas; Gas incubator, Plas, Campylo, CO_2; varied types of AnaeroPack Mean±SD by two plates observed by triplicate wells (two times examination by using one plate).

Table 2 Growth by % parasitaemia with different number of plates in an AnaeroPack box jar at different gas conditions

No. of plate in a box jar	Gas	Plas	Campylo	Candle Jar
One	5.3±0.2%	4.2±0.3%	4.0±0.8%	4.5±0.6%
Five	4.9±0.3%	7.3±1.2%	4.8±0.4%	4.5±0.5%

Mean±SD by two plates observed by triplicate wells (two times examination by using one plate after 48 hr incubation changing media and sachet).

Table 3 Percent (%) concentration of CO_2 and O_2 in a box jar with 1 or 5 plates after 24 hr incubation

Concentration	Gas	Plas	Campylo	Candle Jar
CO_2 with 1 plates (%)	4.0	5.3	6.0	4.0
CO_2 with 5 plates (%)	4.0	4.5	6.4	6.0
O_2 with 1 plate (%)	5.0	6.4	12.7	16.0
O_2 with 5 plates (%)	5.0±0.7	4.8	11.2	14.0±2.0

Mean by double experiments.

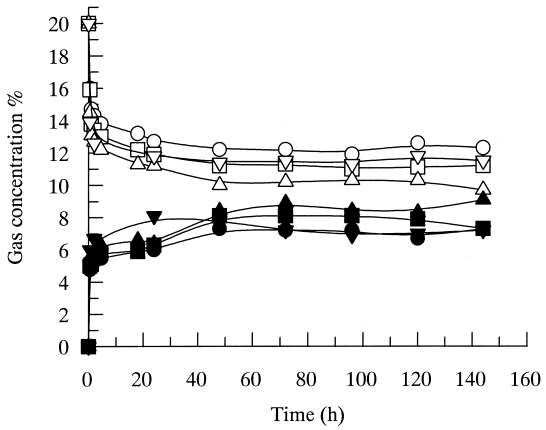

Figure 2 Gas concentration with different number of plates using AneroPack Campylo. There are four conditions, without plate, with single, three or five plates in an AnaeroPack box jar. AnaeroPack sachets in a Jar were renewed every 24 hr at the time of sampling. Open figures indicate O_2 concentrations and closed figures indicate CO_2. The shapes of figures indicate the number of the plates as follows: circles with single plate, square with three plates, triangles with five plates and inverted triangles without plate.

dle jar as they are not enclosed in a small space. The change of free space in a Jar also modified gas concentrations a little in AnP methods. The concentrations of CO_2 are not changed much in AnP methods as in gas incubator and candle jar with some variations. The concentrations of O_2 however, does not go down to 10% with AnP Campylo with a single plate but far better with 5 plates, whilst AnP Plas with 5 plates approached to the gas composition as in a gas incubator after 24 hr.

The time course of changes in gas composition in a jar was observed using AnP Campylo when none, 1, 3, or 5 plates were placed in a Box Jar. It took about 24 hr until gas composition became stable in a jar, and concentrations of CO_2 and O_2 were modified and became more stable when 5 plates were placed (Fig 2).

Generally, high altitude with low atmospheric pressure hinders the growth of *P. falciparum* in the culture (Wernsdorfer and Payne, 1988). It is feasible to speculate that the chemical reaction of AnPs may interfere the internal pressure of a Box Jar. AnPs absorb oxygen and produce carbon dioxide in making a different ratio of gas composition finally. Therefore, the internal pressure of the Jar is one of the determinants to affect the culture when different AnPs are used. In this respect, the pressure difference inside of the Jar from the outside was measured, and it showed negative with AnP Plas and further decreased from -14 to -20 Hecto Pascal (HP) with more plates, whereas AnP Campylo showed slightly positive. By the results with AnP Plas, the pressure was changed as an equivalent altitude of about 150 m above sea lebel (Table 4). The change was minor and can be ignored for the parasite culture.

It is clear that the AnPs are a good tool for the parasite culture by these experiments. It is considerable that the difference of gas concentrations and pressures affect the results of IC_{50} of CQ. For determining reliable IC_{50}, the preferable gas concentrations are 3% O_2 and 4% CO_2 as in a gas phase incubator. It is important to compare these AnP types by evaluating the observed IC_{50} figures for its reliability and immutability. In terms of CQ sensitivity assay, difference of IC_{50} of K1 was obvious in different gas phases. IC_{50} of K1 in a gas incubator was 217±16 nM whilst those by AnPs Plas, Campylo (no significant difference), CO_2 and in Candle Jar (significant difference between ordinary gas method. p=0.007) were 269±15 nM, 235±38 nM, 149±18 nM and 137±23 nM, respectively (Table 5).

Table 4 Difference of atmospheric pressure inside of a box jar from outside when placing 1 or 5 plates in a Jar after 24 hr incubation

Number of plates	Plas	Campylo
1 plate	-14HP	+2HP
5 plates	-20HP	+4HP

Single experiment. HP=Hecto Pascal.

Table 5 IC_{50} of K1 strain at different gas conditions and with 1 or 5 plates in a box jar

	Gas	Plas	Compylo	CO_2	Candle Jar
IC_{50} (nM) with 1 plate	217±16	269±15	235±38	149±18	138±23
IC_{50} (nM) with 5 plates	220±22	202±36	217±44	ND	122±34

Mean±SD by two plates observed by triplicate wells.

By the results observed IC_{50}, gas phases can be divided into two groups. The one involves gas incubator, AnP Plas, and AnP Campylo, and the other does the Candle Jar and AnP CO_2. The free space also affects the results of IC_{50}. With both Campylo and Plas, IC_{50} levels were lowered with five plates than a single plate, indicating a little more sensitive to CQ in a narrower free space in a Jar. Only using AnP plas and ordinary gas method showed significant difference with single plate ($p=0.99$) but not with five plates. Of course, there is no difference in ordinary gas methods by the different number of plates.

DISCUSSION

Since establishment of *in vitro* culture of *P. falciparum* (Trager and Jensen, 1976), drug sensitivity assay has been introduced by WHO (Bruce-Chwatt *et al.,* 1986). Although CQ resistant parasite has been distributed world widely, it is not well known quantitatively how widely and deeply it is spreading now in the world. We need to monitor the drug sensitivity in the field widely in terms of drug choice for prevention and treatment.

WHO standard test is based on the candle jar method (Rieckmann *et al.,* 1978). Therefore, it has been so far the only method to culture the parasites for drug sensitivity tests in the field. But candle jar is labour intensive and difficult to manage. Usually, gas incubator or gas chamber is used for laboratory works only in the well equipped laboratories for malaria experiments, drug sensitivity tests inclusive (Desjardins *et al.,* 1979). Initially, Trager and Jensen (1976) suggested that the culture of the parasite was good at low O_2 (5%) and high CO_2 (7%). They also suggested that the candle jar condition is also possible to culture the parasite in the field alternatively, because of a lack of equipment in developing countries. But the other hand, Desjardins *et al.* (1979) established a drug sensitivity test using radioactive hypoxanthine in laboratory at gas concentrations of 5% O_2 and 5% CO_2. This method nowadays becomes a standard laboratory method for drug sensitivity test world widely. Although the gas condition affects on the results of CQ sensitivity test, 2 different gas conditions are used for drug sensitivity test at moment. That is why there is a discrepancy of the results between field and laboratory CQ sensitivity tests. The authors tried to characterise AnP system in terms of parasite growth of malaria and CQ sensitivity test for considering a stable method other than candle jar, and eliminating a discrepancy of the results in the laboratory and field.

We reported for the first time that AnP Campylo was producing appropriate gas phase for malaria cultivation (Haruki *et al.,* 1997a, b, 1998). Soon after, Taylor-Robinson (1998) submitted a short paper regarding this method without using a specific jar regardless of gas composition and pressure in the jar.

The AnP system was originally used for anaerobic bacterial culture by producing CO_2 and absorbing O_2 from gas phase to maintain a suitable condition for anaerobic bacterial culture by enclosing them in a specific AnP Box Jar. There are three types of products, *i.e.,* AnP CO_2, AnP Campylo and AnP Plas. The suitable gas conditions of cultivating the parasite is known to be 2-5% CO_2 and 3-18% O_2 (Jensen 1988). In fact, recent studies showed the O_2 concentration above 5% also acceptable conditions using AnP Campylo (Haruki *et al.,* 1997a, b, 1998). Onda *et al.* (1999) reported use of AnP CO_2 to maintain the parasite growth for 26 days.

Although all AnP systems showed fairly good results for the growth of parasites, the number of incubated plates or the volume of free space in a jar affected the results of the growth with AnP plas, because varied volume of free space in a jar changed the gas concentration and pressure in it by the following reasons;

AnP Plas ideally maintains 5% of both O_2 and CO_2 Hence, a total of 15% volume of O_2 is absorted by AnP Plas from enclosed air, and it produces 5% volume of CO_2 by chemical reaction. The total balance of volume will be minus 10%. This chemical reaction changed internal pressure negatively. The other hand, AnP Campylo maintains 10% of both O_2 and CO_2 and balance of the gas volume maintained as minus 10% volume of O_2 and plus 10% volume of CO_2 subsequently. Therefore, internal pressure also maintained as same as the original pressure. By placing more plates in a Jar, AnP Plas absorbs a restricted amount of gas, which was calculated and anticipated by the maker using the box jar, if free space is narrower, and the initial volume of total gas is smaller. Once chemical reaction starts, absorbed restricted amount of gas interferes the internal pressure more prominently than in the wider free space. Because of the equal balance of the gas volumes taken and released by AnP Campylo, internal pressure will be maintained nearly at the original level even in a wider space as same as in a narrower space. Whilst five plates stabilised gas composition at 140 hr, single plate did not stabilised gas composition as product data even 140 hr incubation. This indicates that AnPs are not exactly same as laboratory method and will be an alternative method to culture and drug sensitivity assay. AnPs will be acceptable method in the field but not in laboratory, because of its stability variation.

CQ sensitivity test using AnP CO_2 was also studied by Lin *et al.* (1999). As well as in that report, our results also indicated that the IC_{50} levels with AnP showed the results

similar to those in the candle jar, as gas conditions, by AnP CO_2 appear to be similar in the candle jar, because AnP CO_2 was intentionally developed as a replacement of the candle jar for anaerobic bacterial culture.

A Candle Jar is heavy and difficult to carry, easily brealable and occupies a large space in an incubator whereas the AnP Box Jar is small in size and light weighing only 500 g. Hence the drug sensitivity test using AnP system in an AnP Box Jar will be a set of equipment which is easily portable in the malarious countries.

In the results with AnP Plas, although the pressure was changed as an equivalent altitude of about 150 m above sea level, the change was minor and can be ignored for the parasite culture. Proper condition of malaria cultivation using AnP Plas in consideration with drug sensitivity test would be putting totally 5 culture including dummy plates placed in an AnP Box Jar, if the testing plates were less than 5 (One plate occupies the space of 166 cm^3, and then five plates occupy 830 cm^3 of the space inside the jar). Because AnP Plas reduce the pressure and AnP Campylo can not produce the stable gas concentration with wider space. For drug sensitivity test, if the gas phase common to the gas incubator is required, AnP Plas or AnP Campylo is useful, but the latter will be slightly superior in keeping positive pressure inside the Jar and resulting in better parasite culture. The different pressure may affect the result of IC_{50} with especially CQ, because CQ is weak base and its accumulation to parasite food vacuole interfered by pH of media (SA Ward personal communication).

If the field CQ sensitivity test following WHO standard is attempted, AnP CO_2 is recommended, since its gas composition and the result of IC_{50} are close to those available in a candle jar by which, however, the result may indicate more CQ sensitive than by other AnPs or in the gas incubator. Finally, to use this system for CQ sensitivity test widely in practice in the field, the method should be studied more in comparing laboratory experiment and WHO standard method, and after accumulating the field experience.

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REFERENCES

1) Bruce-Chwatt, L.J., Black, R.H., Canfield, C.J., Clyde, D. F., Peters, W. and Wernsdorfer, W.H., (1986): Chemotherapy of Malaria, 2nd ed., WHO Geneva.
2) Desjardins, R.E., Canfield, C.J., Haynes, J.D. and Chulay, J.D. (1979): Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. Antimicrobial Agents and Chemotherapy, 63, 710-718
3) Haruki, K., Fujino, T., Yokota, N., Kobayashi, F., Matsui, T. and Tsuji, M. (1997a): Evaluation of new drug sensitivity assay: Can AnaeroPack System be a novel tool to determine drug sensitivity of *P. falciparum*? 9th Malaria Meeting, British Society of Parasitology, Programme and Abstracts, 58-59.
4) Haruki, K., Fujino, T., Yokota, N., Kobayashi, F., Matsui, T. and Tsuji, M. (1997b): Evaluation of AnearoPack Series to detemine drug sensitivity of *P. falciparum.* Jpn, J. Trop. Med., 25, Supplement, 87 (Japanese text)
5) Haruki, K., Kobayashi, F., Fujino, T., Matsui, T. and Tsuji, M. (1998): Evaluation of AnaeroPack Campylo and newly developed AnaeroPack series for culture and drug sensitivity assay of *Plasmodium falciparum.* 9th Int. Cong. Parasitol., Makuhari, Japan. Parsit. Int., 47, Supplement, 379
6) Jensen, J.B. (1988): *In vitro* cultivation of malaria parasites, Erythrocytic stages, Malaria Vol. 1, ed. by Wernsdorfer, W.H. and MacGregor, Sir I., Churchil Livingstone, Edinburgh, 307-347.
7) Lin, Q., Onda, T., Kano, S., Masuda, G. and Suzuki, M. (1999): *In vitro* drug susceptibility test of *Plasmodium falciparum* using a portable thermostat and CO_2 gas. J. Japan. Assoc. Inf. Dis., 73, 1099-1133 (Japanese text)
8) Mizuno, Y., Hatabu, T., Kawazu, S., Masuda, G., Ohtomo, H., Suzuki, M. and Kano, S. (2000): Cultivation of *Plasmodium falciparum* isolates under the AneroPack gas condition in a portable themostatic incubator. Jpn. J. Trop. Med. Hyg., 28, 383-385
9) Onda, T., Lin, O., Kano, S. and Suzuki, M. (1999): Continuous *in vitro* culture of *Plasmodium falciparum* using microanaerophilic gas generators and portable incubator. J. Japan. Assoc. Inf. Dis., 73, 1095-1098 (Japaniese text)
10) Rieckmann, K.H., Sax, L.J., Campbell, G.H., and Mrema, J.E., (1978): Drug sensitivity of *Plasmodium falciparum*-An *in vitro* microtechnique. Lancet, 7, 22-23
11) Taylor-Robinson, A.W. (1998): Cultivation of *Plasmodium falciparum in vitro* using microbiological gas sachets that generate micronaerophilic conditions. Trans. Roy. Soc. Trop. Med. Hyg., 92, 357-358
12) Trager, W. and Jensen, J.B., (1976): Human malaria parasites in continuous culture. Science, 193, 673-675
13) Wernsdorfer, W.H. and Payn, D. (1988): Drug sensitivity test, malaria parasites. Malaria Vol. 2, ed. by Wernsdorfer, W.H. and MacGregor, Sir I., Churchil Livingstone, Edinburgh. 1765-1794

MORPHOLOGICAL RE-EXAMINATION OF *PARAGONIMUS WESTERMANI* DESCRIBED BY DAENGSVANG AND OTHERS IN 1964

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Abstract: We examined specimens of adult *Paragonimus westermani* collected from Thai leopards by Daengsvang *et al.* in 1964. The two flukes examined had a six-lobed ovary each and singly-spaced cuticular spines. The seminal receptacle and seminal vesicle were filled with spermatozoa. Based on morphological features, these flukes were identified as the diploid type of *P. westermani.* The testis branched out into six lobes on the ovarian side and into five on the opposite side in both specimens. Abnormal branching in the testes, that is, separation of one lobe from the others and its direct connection to the vas deferens, which was found in adult *P. westermani* from the Philippines, was undetectable.

Key words: Lung fluke, *Paragonimus westermani,* Diploid, Morphology, Thailand

INTRODUCTION

The lung fluke remained undiscovered in Thailand until 1964, when Daengsvang *et al.* (1964) isolated adult *Paragonimus westermani* from leopards captured in southern Thailand. Soon thereafter, Miyazaki and Wykoff (1965) described a new species, *P. siamensis,* and Miyazaki and Vajrasthira (1967a) reported the occurrence of *P. heterotremus.* Miyazaki and Vajrasthira then described a new species, *P. bangkokensis* (Miyazaki and Vajrasthira, 1967b), the occurrence of *P. macrorchis* (Miyazaki and Vajrasthira, 1967c) and the discovery of another species, *P. harinasutai* (Miyazaki and Vajrasthira, 1968). Thus the number of species of the genus *Paragonimus* identified in Thailand totals six. From a medical viewpoint, *P. heterotremus* is the most important because it infects humans readily and occurs widely in various parts of Thailand (Vanijanonta *et al.*, 1984). However, human infection with *P. westermani* has not been documented in Thailand.

In 1977, Miyazaki reported that lung flukes, regarded as *P. westermani* for almost a century, could be divided into two types according to the contents of the seminal receptacle of the adult specimens: one is the bisexual (or original) type in which the seminal receptacle is filled with sperm and the other is the parthenogenetic (or nonspermatozoan) type in which it is occupied by egg cells and vitelline cells (Miyazaki, 1977). Miyazaki's opinion has its base on the results of the chromosome study of *P. westermani* by Sakaguchi and Tada (1976) and Terasaki (1980). They showed that the bisexual type is diploid with karyotype 2n=22, while the parthenogenetic type is triploid with karyotype 3n=33. Miyazaki (1978) then proposed that the parthenogenetic type should be referred to as a separate species named *P. pulmonalis.* Some researchers, however, have argued against such separate specific naming for the parthenogenetic triploid form of *P. westermani* (Blair *et al.,* 1997).

We were offered the opportunity to observe the specimens of adult *P. westermani* collected from Thai leopards by Daengsvang *et al.* (1964). Re-examination of these specimens revealed that they were diploid, as described below.

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MATERIALS AND METHODS

Adult flukes of *P. westermani* were collected from naturally infected leopards caught in Choomporn Province in southern Thailand (Daengsvang *et al.*, 1964). The two specimens examined in this study were stained with borax carmine and mounted with Canada balsam.

RESULTS

The specimens 1 and 2 (Figs. 1 and 4) measured 13.5 mm and 12.9 mm in length and 8.7 mm and 7.6 mm in width, respectively. Cuticular spines were originally single (Figs. 2 and 5), but some of them had split into two or more at the terminal end. The oral sucker was located near the anterior end of the body, and was 1.32 and 1.21 mm wide in specimens 1 and 2, respectively. The ventral sucker was situated somewhat anteriorly to the middle of the body and was 1.00 and 0.95 mm wide in specimens 1 and 2, respectively. The ventral sucker was slightly smaller than the oral sucker in both flukes. The intestine wound gently and ran posteriorly to the end of the body. The ovary, situated on the anterolateral side of the ventral sucker, was divided into six lobes, each of which was subdivided into short sections (Figs. 3 and 6). The seminal receptacle and seminal vesicle were small but filled with spermatozoa. The uterus was filled with eggs and situated on the opposite side of the ovary. The vitelline glands were widely and bilaterally distributed. The testes, situated at both sides of the posterior part of the body, were almost as large as the ovary. The testis branched out into six lobes on the ovarian side and into five on the opposite side in both flukes. Abnormal branching of the testes was not observed; that is, none of the lobes were separated from the rest and directly connected to the vas deferens.

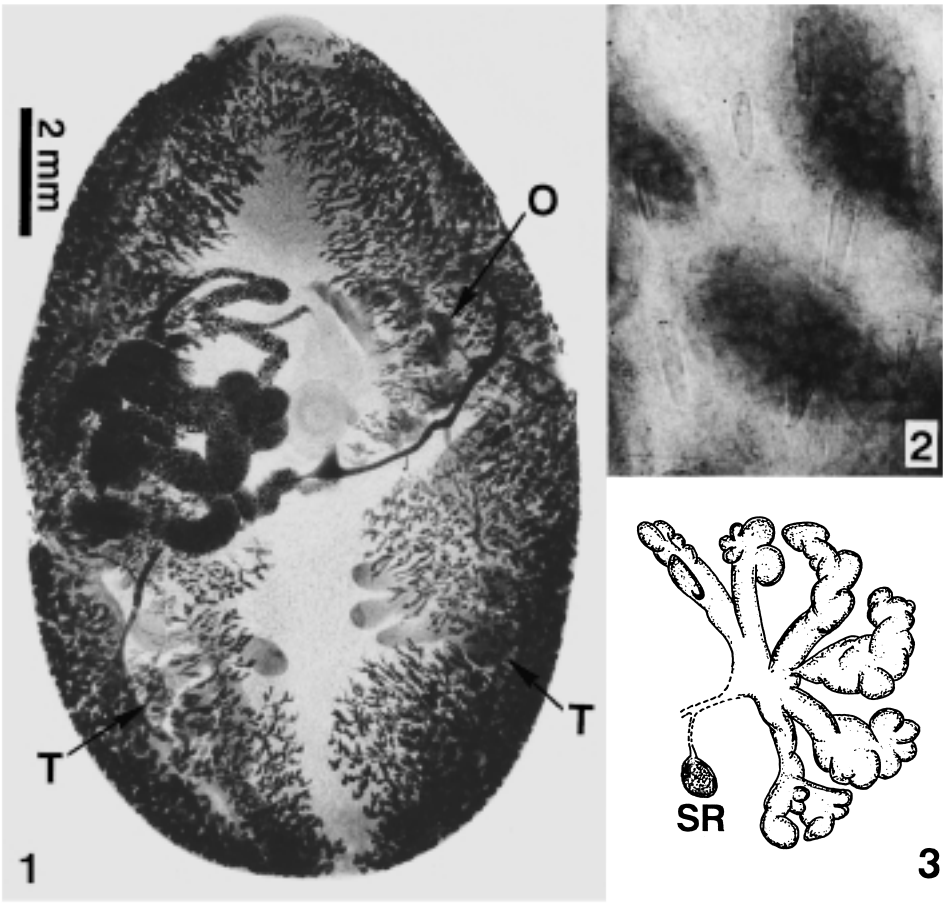

Figure 1 Mounted adult fluke specimen of *Paragonimus westermani,* No. 1, stained with borax carmine, showing the ovary (O) and testes (T).
Figure 2 Microphotograph of cuticular spines, showing singly-spaced arrangement.
Figure 3 Camera lucid drawing of the ovary, which was divided into six lobes. SR, seminal receptacle.

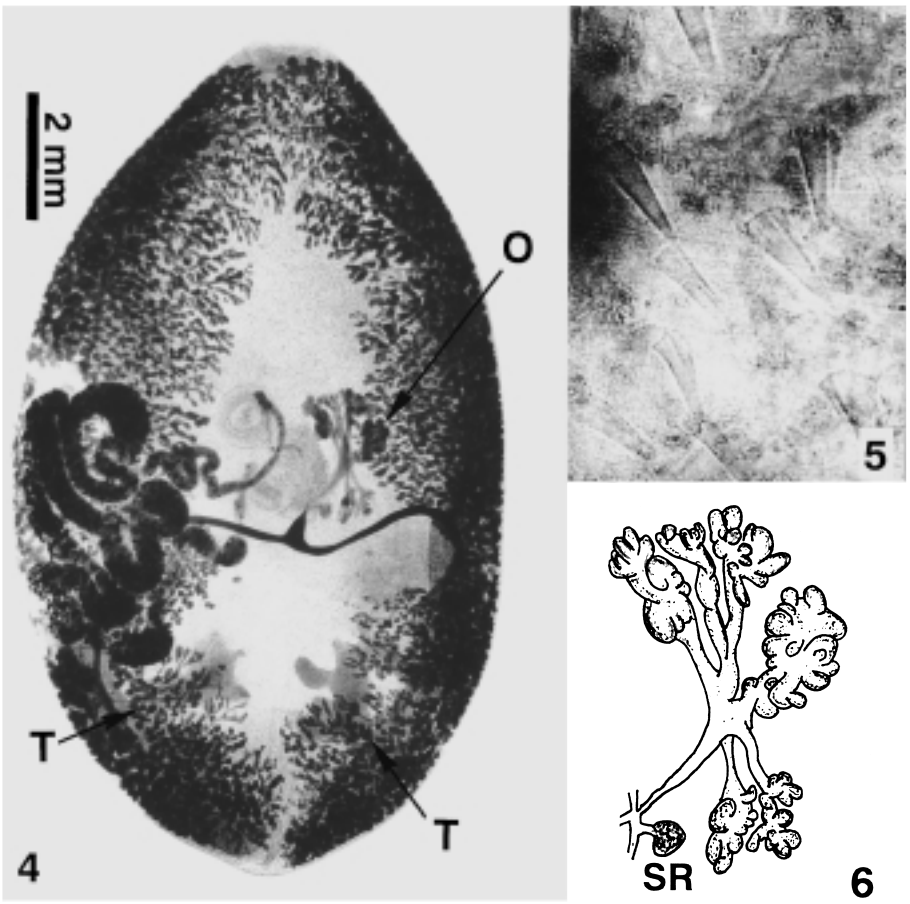

Figure 4 Mounted adult fluke specimen, No. 2, stained with borax carmine, showing the ovary (O) and testes (T).
Figure 5 Microphotograph of cuticular spines, showing singly-spaced arrangement.
Figure 6 Camera lucid drawing of the ovary, which was divided into six lobes. SR, seminal receptacle.

DISCUSSION

Daengsvang *et al.* (1964) isolated adult *P. westermani* from Thai leopards, but the morphological characteristics of the flukes were not described other than that the features of the flukes were identical to the description of *P. westermani* by Yamaguti (1958). In the present study, the adult flukes collected by Daengsvang *et al.* (1964) were examined and were identified as the diploid type of *P. westermani* on the basis of morphological features. The flukes examined closely resembled *P. siamensis* in morphological features, but the cuticular spines of adult *P. siamensis* are arranged in groups all over the body surface (Miyazaki and Wykoff, 1965), whereas the spines were singular in *P. westermani.*

We recently found *Paragonimus* metacercariae in the crab, *Siamthelphusa improvisa,* from Surat Thani Province in southern Thailand. Size and shape indicated that they were *P. westermani* metacercariae. In a scanning electron microscopic study, Kanla *et al.* (1998) observed excysted metacercariae of *P. westermani* harvested from crabs in Nakhon Nayok Province in central Thailand. Taken together, these findings suggest that the life cycle of *P. westermani* is still maintained in Thailand with crabs functioning as the second intermediate host.

The phylogeny and/or intraspecific variation of *P. westermani* have been studied using nuclear ribosomal DNA {second internal transcribed spacer (ITS2)} and mitochondrial DNA (partial COI) sequences (Blair *et al.,* 1997; Iwagami *et al.,* 2000). The results indicated that *P. westermani* could be partitioned into at least 2 groups (or species): the northeastern Asian group and the southern Asian group. The former group consisted of both diploid and triploid types of *P. westermani* from China, Korea, Japan and Taiwan and the latter consisted of *P. westermani* from Malaysia, the Philippines and Thailand, which were confirmed or assumed to be diploid. These two groups were also distin-

guished by the difference in snail host specificity. *P. westermani* in the northeastern group utilized the pleurocercid snails as the first intermediate hosts, whereas those in the southern group utilized the thiarid snails (Davis *et al.*, 1994). Even within the groups, however, the morphological features of the adult flukes were not universal. As Miyazaki (1981) described, adult *P. westermani* from the Philippines exhibited some morphological peculiarities in the testes; one or two lobes of one or both testes were often separated from the rest and directly connected to the vas deferens. This abnormal branching was observed in 83 (18.4%) of 450 adult flukes examined. Miyazaki (1978) also examined at least 36 adult flukes of *P. westermani* from Malaysia but did not mention such abnormalities in those specimens. Abnormal branching was undetectable in the testes of *P. westermani* from Thailand in the present study. More specimens from various localities in Thailand should be investigated to confirm our findings.

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REFERENCES

1) Blair, D., Agatsuma, T., Watanobe, T., Okamoto, M. and Ito, A. (1997): Geographical genetic structure within the human lung fluke, *Paragonimus westermani*, detected from DNA sequences. Parasitology, 115, 411-417
2) Daengsvang, S., Papasarathorn, T. and Tongkoom, B. (1964): *Paragonimus westermani* (Kerbert, 1878) in Thai leopards. Ann. Trop. Med. Parasitol., 58, 304-306
3) Davis, G.M., Chen, C.E., Kang, Z.B. and Liu, Y.Y. (1994): Snail hosts of *Paragonimus* in Asia and the Americas. Biomed. Env. Sci., 7, 369-382
4) Iwagami, M., Ho, L.Y., Su, K., Lai, P.F., Fukushima, M., Nakano, M., Blair, D., Kawashima, K. and Agatsuma, T. (2000): Molecular phylogeographic studies on *Paragonimus westermani* in Asia. J. Helminthol., 74, 315-322
5) Kanla, P., Yaemput, S., Arunyanart, C. and Maleewong, W. (1998): Scanning electron microscopy of newly excysted metacercariae of *Paragonimus westermani*-like, Nakhon Nayok, Thailand. Southeast Asian J. Trop. Med. Public Health, 29, 748-750
6) Miyazaki, I. (1977): A newly introduced question on *Paragonimus westermani* (Kerbert, 1878). Jpn. Med. J., 2788, 43-46
7) Miyazaki, I. (1978): Two types of the lung fluke which has been called *Paragonimus westermani* (Kerbert, 1878). Med. Bull. Fukuoka Univ., 5, 251-263
8) Miyazaki, I. (1981): Taxonomical studies on the lung fluke occurring in the Philippines. Med. Bull. Fukuoka Univ., 8, 153-158
9) Miyazaki, I. and Vajrasthira, S. (1967a): Occurrence of the lung fluke *Paragonimus heterotremus* Chen et Hsia, 1964, in Thailand. J. Parasitol., 53, 207
10) Miyazaki, I. and Vajrasthira, S. (1967b): On a new lung fluke, *Paragonimus bangkokensis* sp. nov. in Thailand (Trematoda: Troglotrematidae). Jpn. J. Med. Sci. Biol., 20, 243-249
11) Miyazaki, I. and Vajrasthira, S. (1967c): Occurrence of the lung fluke *Paragonimus macrorchis* Chen, 1962 in Thailand. J. Parasitol., 53, 894-895
12) Miyazaki, I. and Vajrasthira, S. (1968): On a new lung fluke found in Thailand, *Paragonimus harinasutai* sp. nov. (Trematoda, Troglotrematidae). Ann. Trop. Med. Parasitol., 62, 81-87
13) Miyazaki, I. and Wykoff, D.E. (1965): On a new lung fluke, *Paragonimus siamensis* n. sp. found in Thailand (Trematoda, Troglotrematidae). Jpn. J. Parasitol., 14, 251-257
14) Sakaguchi, Y. and Tada, I. (1976): Chromosomes of a lung fluke, *Paragonimus westermani*. Chromosome Inf. Serv., 20, 23-24
15) Terasaki, K. (1980): Comparative studies on the karyotypes of *Paragonimus westermani* (s. str.) and *P. pulmonalis.* Jpn. J. Parasitol., 29, 239-243
16) Vanijanonta, S., Bunnag, D. and Harinasuta, T. (1984): *Paragonimus heterotremus* and other *Paragonimus* spp. in Thailand: pathogenesis, clinic and treatment. Arzneim. Forsch., 34, 1186-1188
17) Yamaguti, S. (1958): Paragonimidae Dollfus, 1939, Yamaguti, S., (ed.), Systema Helminthum, Vol. 1, The digenetic trematodes of vertebrates. Part 1, 931-933, Interscience Publishers, New York

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SURFACE ULTRASTRUCTURE OF NEWLY EXCYSTED METACERCARIAE OF *PARAGONIMUS WESTERMANI* FROM MALAYSIA AND THE PHILIPPINES

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Abstract: We studied the ultrastructure of excysted metacercariae of *Paragonimus westermani* from Malaysia and the Philippines using scanning electron microscopy. The body surface of the excysted metacercariae was covered with numerous single-pointed and thorn-like tegumentary spines. Dome-shaped papillae were evenly distributed over the whole body and situated circularly around the oral and ventral suckers. Between six to eight and seven to nine small dome-shaped papillae were observed around the ventral sucker in *P. westermani* from Malaysia and the Philippines, respectively. In general, the morphological features of the worms from Malaysia and the Philippines were identical.
Key words: Lung fluke, *Paragonimus westermani*, Scanning electron microscopy, SEM, Ultrastructure, Malaysia, the Philippines

INTRODUCTION

The external appearance of newly excysted metacercariae has been studied on various species of the genus *Paragonimus* using scanning electron microscopy (SEM; Higo and Ishii, 1984, 1987; Sugiyama *et al.*, 1990). The number of papillae around the oral and ventral suckers have been reported to vary among species. Moreover, even within a species, for example *P. mexicanus*, the number of papillae around the ventral sucker differed by geographical distribution (Aji *et al.*, 1984; Tongu *et al.*, 1985, 1987, 1995).

Recently, Kanla *et al.* (1998) studied the surface morphology of newly excysted metacercariae of *P. westermani* from Thailand using SEM. They noted that the morphological features of flukes from Thailand and Japan were nearly identical. In the present study, we used SEM to examine the surface ultrastructure of excysted metacercariae of *P. westermani* from Malaysia and the Philippines and to determine whether a geographical difference exists in morphology in *P. westermani*, similar to that seen in *P. mexicanus*.

MATERIALS AND METHODS

P. westermani metacercariae were harvested from the crabs, *Parathelphusa maculata*, from the Kuala Pilah area in Negri Sembilan State of peninsular Malaysia in July, 1985, and, *Sundathelphusa philippina*, from the Jaro area in Leyte Province of the Philippines in September, 1985. The *P. westermani* populations occurring in these localities were identified as the diploid type based on the morphological features of the adult flukes which were recovered from the cats given the metacercariae. Fifteen metacercariae each were available for the present study.

The methods for excystation of *Paragonimus* metacercariae have been previously described (Higo and Ishii, 1984). Excysted metacercariae were washed in 0.1M phosphate buffer (pH 7.4) several times, fixed for 4 hr in 1% phosphate-buffered glutaraldehyde and fixed for 2 hr in 1% phosphate-buffered osmium tetroxide. The specimens were dehydrated in a graded series of ethanol, substituted with isoamyl acetate and critically dried. The metacercariae were mounted on aluminum stubs, coated with platinum-palladium and examined under a scanning electron micro-

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scope (Hitachi S-800) at an accelerating voltage of 5 kv.

RESULTS

P. westermani from Malaysia

The excysted matacercariae had various shapes, although the body of most specimens was flattened dorsoventrally (Fig. 1A). The body surface was covered with numerous single-pointed and thorn-like tegumentary spines. Dome-shaped papillae were evenly distributed over the whole body.

The oral sucker had an inner diameter of about 20 μm and was situated near the anterior end of the worm body (Fig. 1B). Around the oral sucker, three types of dome-shaped papillae were observed: large papillae with a ragged surface, small papillae with an irregular shaped pit and small papillae with a smooth surface. The small dome-shaped papillae with a pit were distributed mainly on the dorsal or dorsloateral side of the sucker and numbered between 25 and 30. The small dome-shaped papillae with a smooth surface were situated on the dorsal or dorsolateral side of the sucker and numbered around 20.

The ventral sucker had an inner diameter of about 40 μm and was situated somewhat anterior to the middle of the worm body (Fig. 1C). Around the ventral sucker, we observed a circular distribution of large dome-shaped papillae internal to small dome-shaped papillae. The number of large papillae was fixed at six, whereas the number of small papillae varied from six to eight.

P. westermani from the Philippines

No distinct difference was evident between *P. wester-*

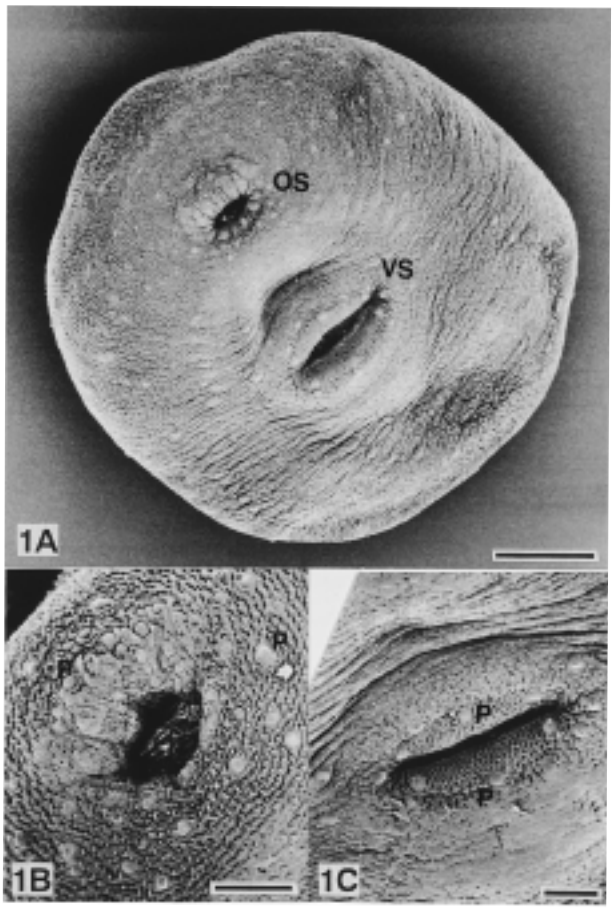

Figure 1 Scanning electron microscopy of newly excysted metacercaria of *P. westermani* from Malaysia.
1A Whole body in ventral view. Bar=30 μm.
1B High power magnification of oral sucker region showing the papillae. Bar=10 μm.
1C High power magnification of ventral sucker region showing the papillae. OS, oral sucker; VS, ventral sucker; P, papillae. Bar=10 μm.

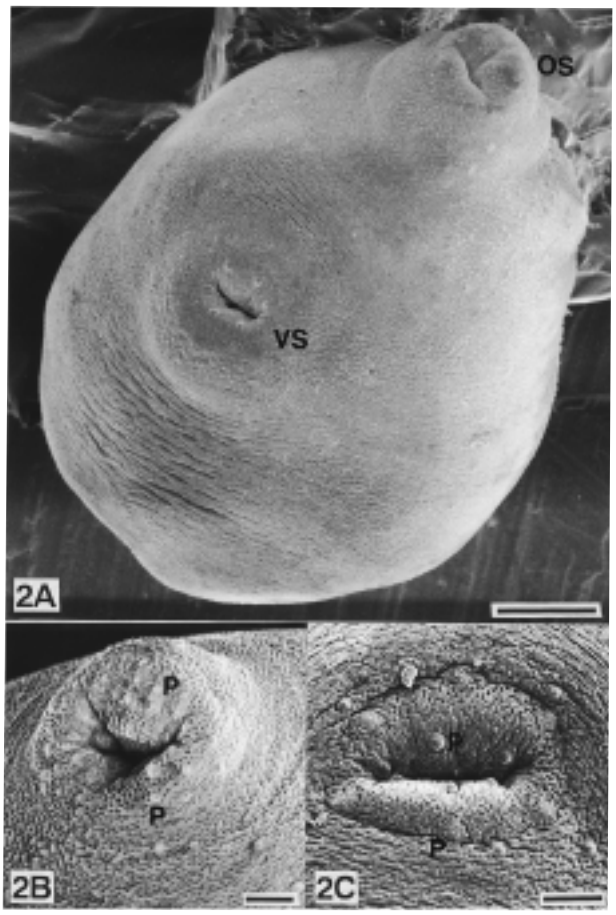

Figure 2 Scanning electron microscopy of newly excysted metacercaria of *P. westermani* from the Philippines.
2A Whole body in ventral view. Bar=30 μm.
2B High power magnification of oral sucker region showing the papillae. Bar=10 μm.
2C High power magnification of ventral sucker region showing the papillae. OS, oral sucker; VS, ventral sucker; P, papillae. Bar=10 μm.

mani from the Philippines and Malaysia either in the shape of metacercarial bodies, in the shape and position of each sucker or in the shape and distribution of the tegumentary spines (Fig. 2A). Dome-shaped papillae also showed similar shape and arrangement to those in the flukes from Malaysia (Fig. 2B). However, the number of papillae around the ventral sucker differed slightly. As seen in *P. westermani* from Malaysia, we observed six large dome-shaped papillae in *P. westermani* from the Philippines. However, the small dome-shaped papillae varied in number from seven to nine (Fig. 2C).

DISCUSSION

The number of small dome-shaped papillae around the ventral sucker of newly excysted metacercariae of the genus *Paragonimus* from Japan varies with the species (Higo and Ishii, 1984, 1987): zero to six papillae are found in *P. ohirai* (zero to two in the metacercaria which has no inner cyst wall and was originally described as *P. iloktsuenensis*, and two to six in the metacercaria which has inner cyst wall), five to 13 in *P. westermani* (five to 11 in the triploid type of *P. westermani* and 10 to 13 in the diploid type), and 13 to 19 in *P. miyazakii*. Moreover, in *P. mexicanus*, a marked geographical difference is observed in the number of these papillae: 22 to 25 papillae are found in *P. mexicanus* from Peru (originally referred to as *P. peruvianus*; Aji *et al.,* 1984), 20 to 30 in those from Guatemala (Tongu *et al.,* 1995), 30 to 35 in those from Mexico (Tongu *et al.,* 1985), and 28 to 38 in those from Costa Rica (Tongu *et al.,* 1987). In the present study, we found that the small dome-shaped papillae around the ventral sucker of excysted metacercariae of *P. westermani* numbered six to eight and seven to nine in Malaysian and Philippine *P. westermani*, respectively. In contrast, the papillae numbered 10 to 13 in *P. westermani* metacercariae found in Thailand (Kanla *et al.,* 1998). Although the number of the papillae varied between *P. westermani* from Thailand and Malaysia, and the Philippines, the variation was small and was within the range of the observed papillae in *P. westermani* from Japan. Our data suggest that a geographical difference does not exist in morphology of excysted metacercariae of *P. westermani* and thus, the number of the papillae (5 to 13) could be used as a criterion for identifying *P. westermani* in the metacercarial stage.

The phylogeny of *P. westermani* has been studied using ribosomal and mitochondrial DNA sequences (Blair *et al.,* 1997; Iwagami *et al.,* 2000). The results indicated that the *P. westermani* examined could be partitioned into at least two groups (or species): the northeastern Asian group and the southern Asian group. The northeastern group consisted of both diploid and triploid *P. westermani* from China, Korea, Japan, and Taiwan, and the southern group consisted of *P. westermani* from Malaysia, the Philippines and Thailand, which were confirmed or assumed to be diploid. Even within each group, however, the internal morphology of the adult flukes was not universal. For instance, as Miyazaki (1981) described, adult *P. westermani* from the Philippines exhibited some morphological peculiarities in the testes: one or two lobes of one or both testes were often separated from the rest and directly connected to the vas deferens. This kind of abnormality was undetectable in adult *P. westermani* from Malaysia (Miyazaki, 1978) and Thailand (Sugiyama *et al.,* unpublished data). However, information on the external morphology of adult *P. westermani* using SEM is still lacking and thus, the potential existence of geographical differences in the surface ultrastructure of *P. westermani* in the adult stage remains to be investigated.

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REFERENCES

1) Aji, T., Oh, H., Tongu, Y., Inatomi, S., Hata, H., Kobayashi, M. Yokogawa, M., Miranda, H. and Ibáñz, N. (1984): Ultrastructure of tegumental surface of the metacercaria of *Paragonimus peruvianus.* Jpn. J. Parasitol., 33,15-21
2) Blair, D., Agatsuma, T., Watanobe, T., Okamoto, M. and Ito, A. (1997): Geographical genetic structure within the human lung fluke, *Paragonimus westermani*, detected from DNA sequences. Parasitology, 115, 411-417
3) Higo, H. and Ishii, Y. (1984): Scanning electron microscopy of the newly excysted juveniles of *Paragonimus westermani* (Kerbert, 1878) Braun, 1899 (parthenogenetic type) and *P. miyazakii* Kamo, Nishida, Hatsushika and Tomimura, 1961. Jpn. J. Parasitol., 33, 421-427
4) Higo, H. and Ishii, Y. (1987): Comparative studies on surface ultrastructure of newly excysted metacercariae of Japanese lung flukes. Parasitol. Res., 73, 541-549
5) Iwagami, M., Ho, L.Y., Su, K., Lai, P.F., Fukushima, M., Nakano, M., Blair, D., Kawashima, K. and Agatsuma, T.

(2000): Molecular phylogeographic studies on *Paragonimus westermani* in Asia. J. Helminthol., 74, 315-322
6) Kanla, P., Yaemput, S., Arunyanart, C. and Maleewong, W. (1998): Scanning electron microscopy of newly excysted metacercariae of *Paragonimus westermani*-like, Nokhon Nayok, Thailand. Southeast Asian J. Trop. Med. Public Health, 29, 748-750
7) Miyazaki, I. (1978): Two types of the lung fluke which has been called *Paragonimus westermani* (Kerbert, 1878). Med. Bull. Fukuoka Univ., 5, 251-263
8) Miyazaki, I. (1981): Taxonomical studies on the lung fluke occurring in the Philippines. Med. Bull. Fukuoka Univ., 8, 153-158
9) Sugiyama, H., Horiuchi, T., Tomimura, T., Shibahara, T., Agatsuma, T., Habe, S., Kawashima, K., Ketudat, P. and Thaithong, S. (1990): Surface ultrastructure of newly excysted metacercariae of *Paragonimus heterotremus.* Southeast Asian J. Trop. Med. Public Health, 21, 109-113
10) Tongu, Y., Aji, T., Oh, H., Ishiii, A., Yokogawa, M., Hata, H., Ito, J. and Lamothe-Argumedo, R. (1985): Surface ultrastructure of *Paragonimus mexicanus* Miyazaki et Ishii, 1968. Jpn. J. Parasitol., 34, 441-447
11) Tongu, Y., Hata, H., Orido, Y., Pinto, M.R., Lamothe-Argumedo, R., Yokogawa, M. and Tsuji, M. (1995): Morphological observations of *Paragonimus mexicanus* from Guatemala. Jpn. J. Parasitol., 44, 365-370
12) Tongu, Y., Iwanaga, Y., Hata, H., Tsuji, M., Yokogawa, M., Morera, P. and Conejo, M. (1987): Morphological features of *Paragonimus* metacercariae from Costa Rica. Jpn. J. Parasitol., 36, 236-241

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