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Review

PATHOLOGY AND PATHOPHYSIOLOGY OF MALARIA

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Abstract: Pathological processes in malaria are the consequence of the erythrocytic cycle of the parasites. Merozoites invade erythrocytes, in which they develop through early trophozoites (ring forms) to late trophozoites and eventually to schizonts. During this process, development of knobs and cytoadherence or rosetting with the knobs play important roles for the falciparum malaria patient to be severely ill. Expression of variant surface neoantigens stimulates the reticuloendothelial system and can cause anemia, tissue hypoxia and cytokine production. Associated fever, paroxysms, headache and other pains are thought to result from cytokines such as interleukins, interferons and tumor necrosis factor released from macrophages or other cells at the time of schizont rupture. In the present paper, pathological and pathophysiological changes mainly in human falciparum malaria are reviewed, emphasizing the importance of basic research to "roll back" the emerging trends of malaria.

Key words: cerebral malaria, *Plasmodium falciparum,* pathology, pathophysiology

INTRODUCTION

Malaria remains the most important of the tropical diseases; in fact, it is re-emerging in areas where it was once eradicated. Controlling malaria is proving to be more and more difficult with the increasing resistance of parasites and mosquitoes to drugs and insecticides. The world malaria situation was discussed in 1998 in Birmingham, UK, among policymakers of the G8 nations, and it has remained one of the key issues in the G8 development focus. The summit discussions, 3 days before Gro Harlem Brundtland's election, enabled the leaders of the G8 countries to offer strong support for the new WHO "Roll Back Malaria (RBM)" initiative, announced on 13 May 1998 by Brundtland herself as the new director general of WHO. The main emphasis of the initiative is to strengthen health services so that effective treatment and prevention strategies are accessible to all individuals who need them. It aims to apply every existing tool effectively, building on experiences of the past-failures and successes alike. An effective vaccine should be one of the best means for controlling malaria, and development of such a vaccine will depend upon precise scientific knowledge of the disease. Basic research must be stressed, particularly research into the pathology and pathophysiology of the disease.

ULTRASTRUCTURE OF THE PARASITIZED ERYTHROCYTE

The *Plasmodia* merozoite is pear-shaped and approximately 1.5 μm in length, with an apical end that has a polar ring and rhoptries. Entry of the merozoite into the erythrocyte begins when the apical end comes close to the host cell membrane. It has been revealed that the apex has a positive electrical charge facilitating attachment to the negatively-charged erythrocyte membrane (Seed *et al.,* 1974). After the contact, the erythrocyte membrane is thickened and forms a junction with the plasma membrane of the merozoite (Fig. 1-a). Merozoite protein is released by the rhoptries and forms a deep pit in the red blood cell. The merozoite then enters the cell maintaining a contact-ring; i.e., the moving junction formed between the thickened membrane of the erythrocyte and the merozoite (Figs. 1-b and -c) (Aikawa *et al.,* 1978). A small projection still connects the apical end of the merozoite and the erythrocyte membrane (Fig. 1-c). When entry is complete, the red cell membrane seals itself; the merozoite is then located within an invagination of the erythrocyte (Fig. 1-d).

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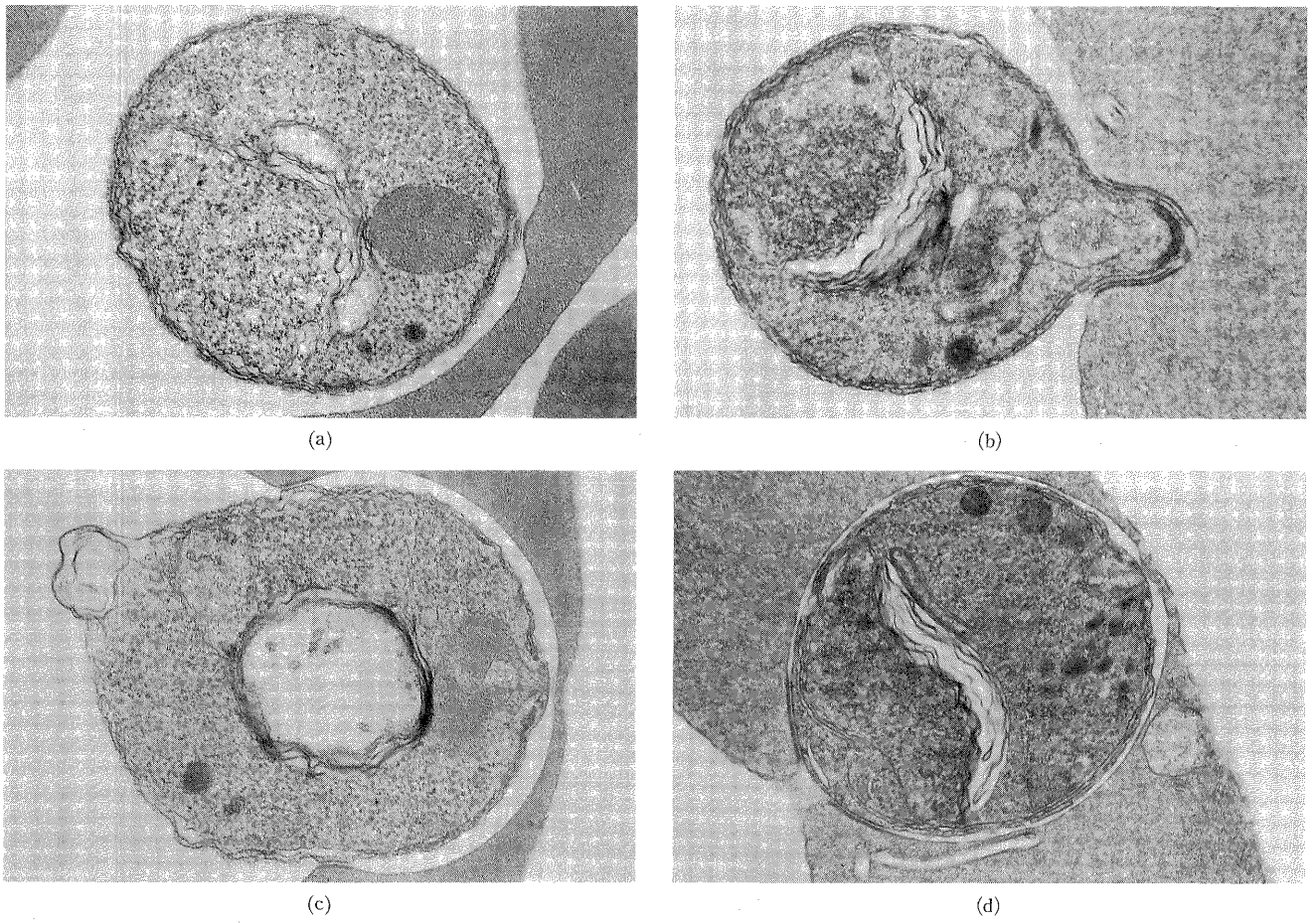

Figure 1 Electron photomicrographs of longitudinal sections of merozoites entering into erythrocytes. (a) Contact between a merozoite and an erythrocyte. The dark part of the merozoite near the junction is the rhoptry. (b) A merozoite just starting to enter into an erythrocyte. (c) A merozoite entering into an erythrocyte at an advanced stage of the interiorization process. Note the moving junction formed between the thickened membrane of the erythrocyte and the merozoite. (d) Completion of the entry of a merozoite into an erythrocyte. The merozoite is left within a vacuole lined with the erythrocyte membrane.

Merozoites develop in the erythrocytes from ring forms to late trophozoites and eventually to schizonts. During the late trophozoite stage, *Plasmodium falciparum* (*P.f.*)-infected erythrocytes start to develop protuberances or knobs on the surface of their cell membrane. Scanning electron micrography has demonstrated numerous, blunt, cone-shaped, protruding knobs evenly distributed over the erythrocyte's entire surface (Aikawa *et al.*, 1983). The same knobs have also been observed on *P. coatneyi*-infected Japanese macaque erythrocytes (Fig. 2) (Kawai *et al.*, 1993). It is now known that the knob consists of a series of antigenically variant erythrocyte-membrane proteins, such as PfEMP1, which mediate binding of the infected erythrocytes to vascular endothelium and uninfected red blood cells (Fujioka and Aikawa, 1996).

Atomic Force Microscopy of the Knob

Atomic force microscopy (AFM), invented by Binning, Quate and Gerber in 1986, has been used in biological research in recent years (Edstrom *et al.*, 1990; Lal and John, 1994). The advantage of AFM is that specimens can be observed without chemical fixation, and the attached surface potential spectroscope can detect surface potential (Yokoyama and Inoue, 1994). When AFM was used to investigate the structure of the erythrocyte knobs, each knob was found to consist of two distinct subunits, knob components that have never been seen in chemically-fixed knobs examined by conventional transmission electron microscopy (Fig. 3). Surface potential spectroscopy revealed that the knobs have a positive charge (+20 mV), whereas the remain-

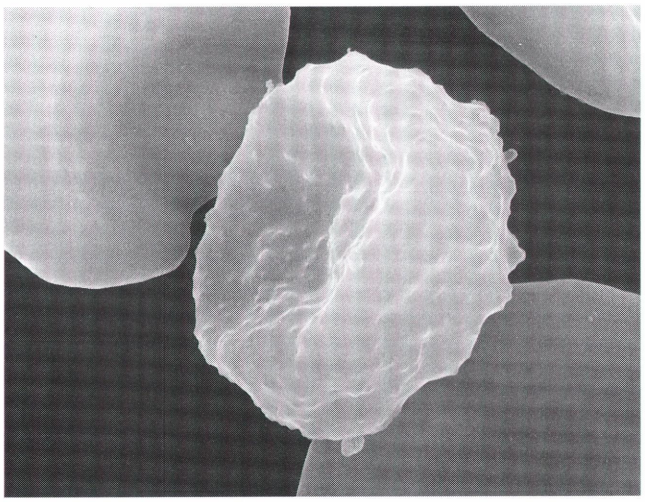

Figure 2 Scanning electron micrograph of a *P. coatneyi*-infected erythrocyte (reproduced courtesy of Dr. S. Kawai, Department of Medical Zoology, Dokkyo University School of Medicine). Compare the knobby surface of the infected erythrocytes with the smooth surface of surrounding non-infected erythrocytes.

der of the red cell plasma membrane is negatively charged (Aikawa *et al.*, 1996). Since endothelial plasma membranes have a negative charge, the difference in charge between the knobs and endothelium may play a significant role in cytoadherence between the two cell types. A therapy that interferes with the localized positive charge of the knobs will likely be the treatment of choice for severe falciparum malaria.

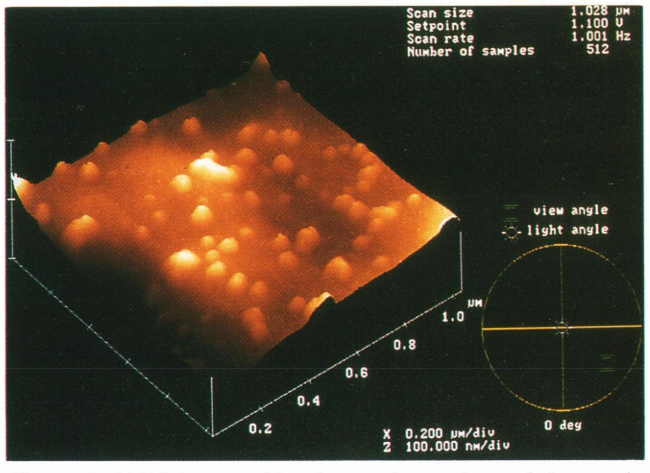

Figure 3 AFM image of knobs on the surface of the erythrocyte in a three-dimensional oblique perspective. Whitish protrusions are knobs with positive electrical charge.

Rosette Formation

Rosette formation or rosetting, the adhesion of non-parasitized erythrocytes to parasitized erythrocytes, is a property exhibited *in vitro* and *ex vivo* in the rat mesoappendix model by some strains of *P.f.* and some sequestering animal malarias (Fig. 4). It has been proposed that rosette formation is a first step to erythrocyte sequestration in blood vessels (Howard and Gilladoga, 1989). An association between human cerebral malaria and erythrocyte rosetting was described based on a study performed in The Gambia (Carlson *et al.*, 1990), in which all isolates from patients with cerebral malaria formed rosettes and in which the mean percentage of parasitized erythrocytes involved in rosetting was twice as high as in the isolates from patients with uncomplicated malaria. Similar findings were also reported from a study in Thailand (Ho *et al.*, 1991), and another investigation conducted in The Gambia showed that giant rosettes formed more frequently in isolates from patients with cerebral malaria than in isolates from patients with uncomplicated malaria (Treutiger *et al.*, 1992).

Scanning electron microscopy revealed that interaction between the rosetted erythrocytes and adjacent uninfected erythrocytes appeared to be mediated by the knobs of parasitized red blood cells (Fig. 5), and transmission electron micrography showed the protruding ends of these knobs to be attached to the membranes of adjacent uninfected erythrocytes (Fig. 6) (Kawai *et al.*, 1995). How, then, do the knobs protrude? It is now known that the binding protein (PfEMP-1) in the knob is encoded by members of the *P.f. var* gene family and

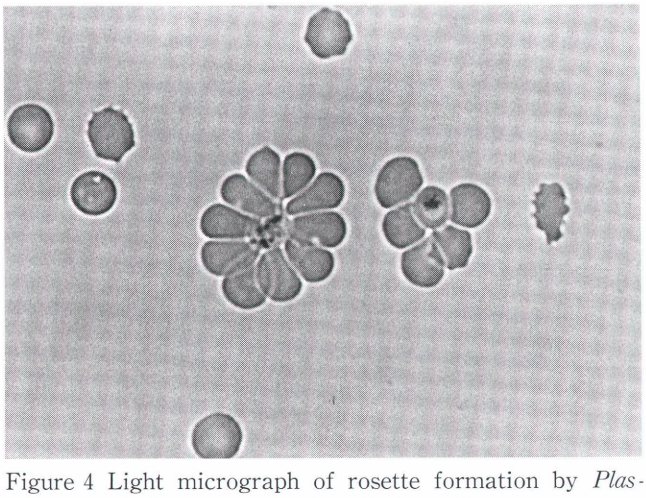

Figure 4 Light micrograph of rosette formation by *Plasmodium coatneyi*-infected erythrocytes of a Japanese macaque, *Macaca fuscata*.

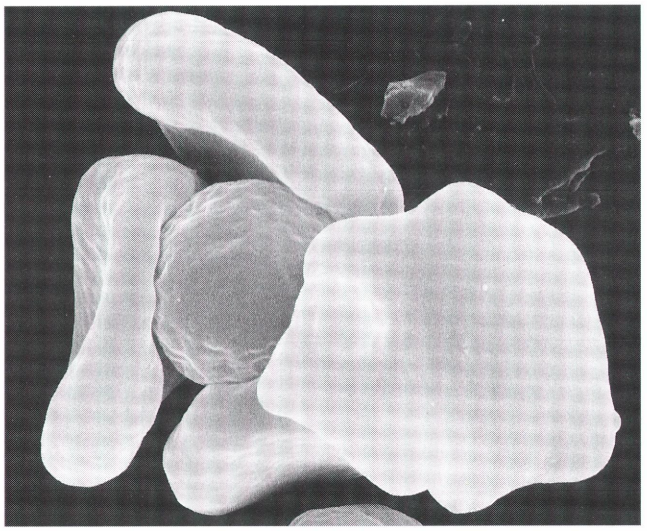

Figure 5 Scanning electron micrograph of a rosette consisting of a central parasitized red blood cell surrounded by several attached uninfected erythrocytes.

that a single *P.f.* parasite simultaneously transcribes multiple *var* genes but, through a developmentally-regulated process, selects only one PfEMP-1 to reach the surface of the host cell (Chen *et al.*, 1999).

A recent report on the formation of rosettes focused on erythrocyte complement-receptor 1 (CR1). It is reported that erythrocytes with a common African CR1 polymorphism, Sl(a$^-$), have reduced adhesion to the PfEMP-1 binding domain, which might have resulted from selection for malaria resistance in highly endemic areas (Rowe *et al.*, 1997).

Pathology of Cerebral Malaria

The brains of some patients who died from cerebral

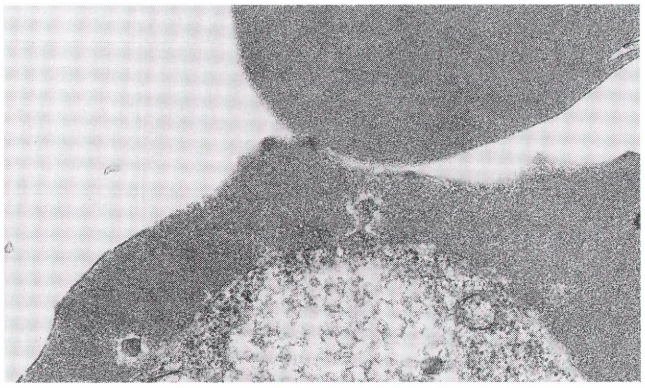

Figure 6 Transmission electron micrograph of a parasitized red blood cell in a rosette attached to an adjacent erythrocyte by electron-dense knobs protruding from the membrane.

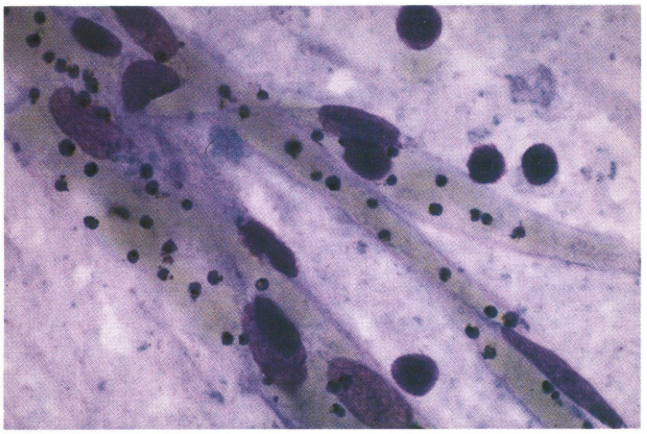

Figure 7 Light micrograph of a Giemsa-stained stamp-smear from post-mortem necropsy brain tissue from a victim of cerebral malaria.

malaria have been described as edematous, but as it is, neither computed tomography (CT) nor nuclear magnetic resonance imaging (NMRI) can demonstrate cerebral edema in situ (Francis and Warrell, 1993). Of course, studies of post-mortem brain tissue limit understanding of the pathology of cerebral malaria. To date, several pathologic changes have been described, and common to all these is the presence of sequestered parasitized erythrocytes undergoing schizogony within microvessels of the brain (Fig. 7). Investigators have reported that the severity of falciparum malaria in humans is proportional to the degree of sequestration (Pongponratn *et al.*, 1991) and not necessarily related to peripheral circulatory parasitemia. The reduced deformability of the parasitized erythrocytes together with their cytoadherence to endothelium and to non-parasitized cells (rosetting) must lead to impaired blood flow. This causes a reduced supply of oxygen and other nutrients to the brain, causing coma. Histological evidence of local inflammatory response to these sequestered vessels is minimal. Infarction, necrosis and large hemorrhages are also rare. However, in many patients, focal ring hemorrhages are seen centered in small subcortical vessels. The pathogenesis of this hemorrhaging has not been clarified, but it may relate to increased capillary permeability.

Electron microscopy has shown that sequestration of parasitized erythrocytes in microvessels is mediated by the knobs (Fig. 8) (Aikawa, 1988; Igarashi *et al.*, 1987). As mentioned above, the molecular basis for cytoadherence is currently of great interest and importance, particularly because of the possibility of preventing this pathogenic process by competitively binding

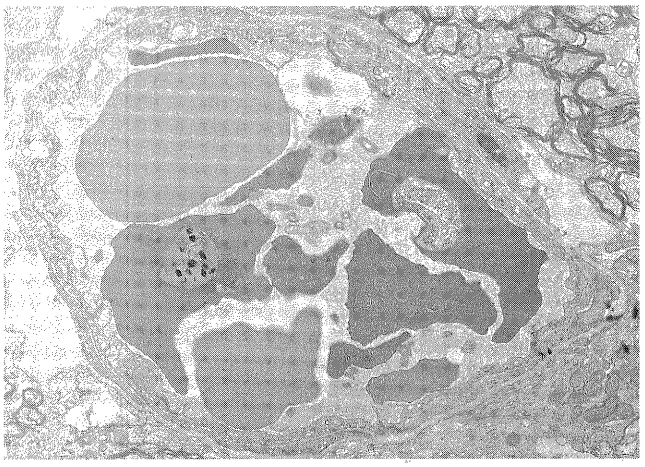

Figure 8 Electron micrograph showing cytoadherence of parasitized erythrocytes to endothelial cells of a cerebral microvessel mediated by electron-dense protruding knobs.

antibodies to the adhesion molecule in the knobs or against receptors on the endothelial cells. The main candidate receptors are CD36, thrombospondin (TSP), intercellular adhesion molecule-1 (ICAM-1), endothelial-leukocyte adhesion molecule-1 (ELAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Aikawa *et al.*, 1990). However, it is quite hard to prove that these molecules are actually playing a role in the cytoadherence of parasitized erythrocytes *in vivo,* because of the difficulty of obtaining fresh tissue from living cerebral malaria patients. Appropriate experimental animal models for human cerebal malaria are thus needed and, to date, *P. coatneyi*-infected rhesus monkeys and Japanese monkeys have been reported as good models (Aikawa *et al.*, 1992; Kawai *et al.*, 1993).

Pathophysiology of Cerebral Malaria

There is increasing evidence in favor of the mechanical hypothesis described above. However, most patients with severe falciparum infection who have recovered from cerebral malaria show no persistent neurological sequelae, which suggests that much of the pathology must be transient and reversible. Therefore, several hypotheses on the events following sequestration have been proposed to explain the development of cerebral malaria.

One of these is the permeability hypothesis, which suggests that increased cerebral vascular permeability causes leakage of plasma across the blood-brain barrier, cerebral edema and eventual coma. Elevated cerebrospinal fluid (CSF) pressure has been reported in African children with cerebral malaria (Newton *et al.*, 1991), which could result from raised intracranial blood volume caused by vasodilation with sequestration of parasitized erythrocytes.

The cytokine hypothesis is based on the observation that plasma concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6 and other cytokines correlate with disease severity (Kwiatkowski *et al.*, 1990). Cytokines released by macrophages and other cells at the time of schizont rupture are thought to be involved in enhancing endothelial cell adhesiveness by increasing the expression of endothelial receptors such as ICAM-1 or CD36. The cytokines can also induce fever, hypoglycemia, coagulopathy, dyserythropoiesis and leucocytosis (Francis and Warrell, 1993).

Cytokines such as IL-1, TNF and lymphotoxin (LT) are reported to be the inducers of nitric oxide (NO), also known as endothelial-derived relaxing factor (EDRF). It is possible that NO contributes in cerebral malaria, through vasodilation of cerebral vessels, to an acute increase in cerebral blood volume, leading to an increase in intracranial pressure and subsequent coma. If vasodilation is caused systemically by increased NO (EDRF) generation, disease severity will be marked by systemic hypotension. It is also possible that NO derived from local endothelial cells diffuses across membranes to influence adjacent neurons as if it were coming from a nearby synapse, disrupting local neurotransmission and thus interfering with neurological function (Clark *et al.*, 1991). A field study conducted in Papua New Guinea revealed an association between high NO levels and disease severity in children with cerebral malaria (Al Yaman *et al.*, 1996). However, report from Tanzania showed NO levels to be inversely related to disease severity, suggesting a protective role (Anstey *et al.*, 1996). The role of NO is now a subject of hot controversy (Holden, 1996) and will be a major focus of upcoming research.

Acknowledgements

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ANTIFILARIAL EFFECT OF *ARTEMISIA NILAGIRICA* EXTRACT AND ITS ULTRA HIGH DILUTIONS AGAINST CANINE DIROFILARIASIS

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Abstract: An ethanolic extract of the flowering meristems of worm wood, *Artemisia nilagirica* was allowed to evaporate. The residue, thus obtained, was administered orally on 4 pariah dogs naturally infected with *Dirofilaria immitis* at 10 mg/kg/day for 15 days and then at 20 mg/kg/day for the next 15 days. Two homoeopathic potencies of the *A. nilagirica* extract, called Cina 200 and Cina 1000, were obtained commercially and administered orally at 0.1 m*l*/dog/day for 30 days on two separate batches, each consisting of 4 dogs. Blood was sampled from the dogs before treatment and on day 15, 30, 45 and 75 following the treatment. *A. nilagirica* extract (Cina θ) was diluted with 90% ethanol 1:100 and shaken by 10 manual strokes to prepare the 1st potency, called Cina 1. All subsequent potencies were prepared by mixing 1 part of the preceding potency with 99 parts of 90% ethanol and giving the mixture 10 manual strokes. Cina θ, Cina 200 and Cina 1000 reduced microfilarial densities in treated dogs by 78.38, 63.06 and 71.40%, respectively on day 30. There were 57.13, 42.44 and 64.20% reduction on day 75. No apparent toxic effect was observed in the treated dogs. Electronic spectra of Cina θ, Cina 200 and Cina 1000 showed comparable absorbance with the latter two giving a blue shift. Cina θ in CCl_4 showed a red shift suggesting molecular complexation and charge transfer (CT) interaction between aqueous ethanol and compounds of *A. nilagirica*. CT was further evidenced by the NMR spectra of the deuterium nuclei of Cina θ in 90% ethanol. NMR spectra of Cina θ, Cina 200, Cina 1000 and 90% ethanol indicated a change in the solution structure of Cina 200 and Cina 1000. This altered solution structure is thought to be responsible for inducing immune reaction of the hosts against the parasite.

Key words: *Artemisia nilagirica,* Antifilarial, *Dirofilaria immitis,* Homoeopathic potency, Ethanol solution structure, Electron transfer

INTRODUCTION

Species of *Artemisia* have long been used by rural people for expelling intestinal nematodes (Singh *et al.*, 1983). High dilutions of the ethanolic extract of the flowering meristems of *Artemisia nilagirica* (Clarke) Pamp have been used in the Homoeopathic system of medicine under the name Cina against intestinal worms (Kent, 1911; Boericke, 1927). The purpose of the present study is to see whether this plant extract, both in its crude form and also its homoeopathic dilutions, called potencies, is effective against canine dirofilariasis.

In Homoeopathy the ethanol extract of *A. nilagirica,* called Cina θ, is diluted with 90% ethanol 1:100 and the mixture is shaken with 10 powerful downward strokes to prepare the first centesimal potency called Cina 1. All subsequent potencies are prepared by adding to one part of the preceding potency 99 parts of 90% ethanol and shaking the mixture in a similar way (Anonymous, 1920; Sukul and Klemm, 1988). Effective homoeopathic potencies could also be produced by sonication instead of mechanical agitation (Sukul *et al.*, 1996; Sukul, 1997). Two potencies of Cina, like Cina 200 and Cina 1000, purchased from King & Co., Calcutta, were used. Since these potencies are too dilute to have any drug molecules, electronic and NMR spectra of them were obtained to find out their difference from the solvent medium like 90% ethanol vis-a-vis the physical basis of their effectiveness. In order to find out the solvent effect on the solute, we prepared Cina θ in a neutral solvent like CCl_4 and obtained the electronic spectra of the solution.

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MATERIALS AND METHODS

Treatment with Cina θ

Cina θ, purchased from King & Co., Calcutta was allowed to evaporate in an incubator at 40°C. The residue was dehydrated in a vacuum dessicator over anhydrous Calcium Chloride and stored at 4°C. Blood was sampled from 4 naturally infected dogs, 2 males and 2 females, every 15 days for a period of 2 months and microfilarial concentration per m*l* of blood was determined. Blood film was allowed to dry, dehaemoglobinised in distilled water and stained with Giemsa stain. The same dogs were then administered orally with the residue of Cina θ at 10 mg/kg body weight/day for 15 days. Blood was sampled from the dogs on day 15. The same dogs were treated again orally with the residue at 20 mg/kg/day for 15 days more. Blood was sampled on day 30, 45 and 75. Capsules were filled with the residue, kept inside a loaf of bread and then offered to the microfilaraemic dogs.

Treatment with Cina 200 and Cina 1000

Blood was sampled from a batch of 4 infected dogs, 2 males and 2 females, every 15 days for 2 months. Cina 200 was mixed with pure cow milk at 0.1 m*l*/4 m*l* of cow milk and 4 m*l* of the mixture was offered in a glass plate to a dog which immediately consumed the mixture. The schedule of treatment and blood sampling were the same as with Cina θ. However, the dosage of Cina 200 was same in the 2 phases of treatment. A batch of 4 naturally infected dogs (3 females and 1 male) was treated with Cina 1000 after determining the mf density for 2 months. The treatment schedule, dosage of the drug and blood sampling were same as with Cina 200.

Electronic spectra of drug

Using a UV-VIS spectrophotometer (Beckman DU 640) absorption spectra of Cina θ in 90% ethanol and Cina θ in carbon tetrachloride, Cina 200 and Cina 1000 against the corresponding solvent blanks were obtained in the wave length range of 190-750 mm at 29°C. The spectra were run in matched quartz cuvettes and were corrected for instrumental baseline errors. Test solutions were kept at the above temperature for at least 10 min to allow for the thermal equilibration.

NMR Spectra of drugs

The spin-lattice relaxation time (T_1 in msec) of the naturally abundant ^2H (0.015%) was measured in 90% ethanol, Cina θ in 90% ethanol, Cina 200 and Cina 1000 using a AMX-400 NMR spectrometer operating at 61.4 MHz at 22°C. The mechanism by which excess spin energy of a nucleus (here ^2H) is shared with the surroundings is referred to as the spin lattice relaxation. The time taken for a fraction 1/e=0.37 of the excess energy to be dissipated is called the relaxation time. Such relaxation comes about by lattice motions like molecular tumbling in liquids (Banwell and McCash, 1994). Deuterium is a quadrupolar nucleus having a small quadrupole moment 1. Quadrupolar relaxation depends upon the interaction of the electric quadrupole moment with an electric field gradient. If the quadrupole moment is small, as it is for ^2H, the interaction is small and the relaxation will be slow. Like all quadrupolar nuclei its relaxation is sensitive to τ_c. τ_c is the average time taken to rotate through 1 radian or roughly the reciprocal of the rate of tumbling of the relevant piece of the molecule (Sanders and Hunter, 1993). T_1 values of ^2H of water, hydroxyl, methylene and methyl groups of ethanol were measured from the stacked spectra with the help of a computer.

RESULTS

Treatment effect on mf count

The mean microfilarial counts per m*l* of blood in 3 batches of dogs before treatment are shown in Table 1. The microfilarial concentration in each dog did not vary appreciably during the 2-month period of observation.

Table 1 Microfilarial concentration/m*l* blood of dogs naturally infected with *Dirofilaria immitis* before treatment

Microfilarial concentration/m*l* blood on different days					
Dogs	Day 0	Day 15	Day 30	Day 45	Day 60
Batch I					
Male	1,001	973	945	997	986
Male	437	423	480	505	440
Female	727	798	756	704	692
Female	813	852	808	826	874
Batch II					
Male	219	241	264	313	274
Male	304	298	253	221	234
Female	535	477	542	580	569
Female	330	374	385	423	450
Batch III					
Male	274	235	252	300	302
Female	236	287	259	247	272
Female	218	269	254	219	203
Female	251	275	244	235	279

Batch I: Treated later with Cina θ
Batch II: Treated later with Cina 200
Batch III: Treated later with Cina 1000

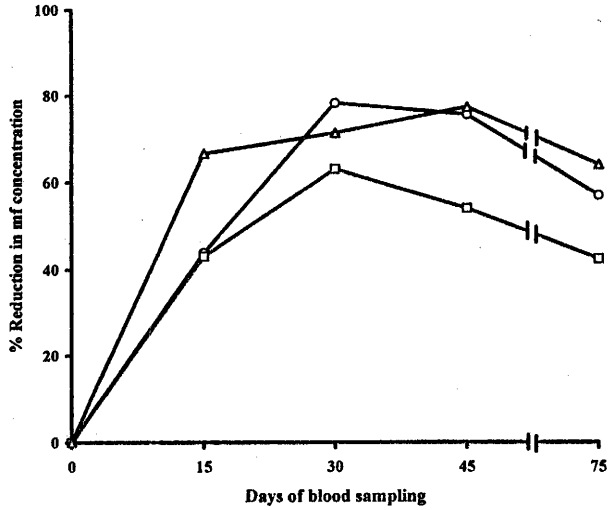

Figure 1 Percentage of reduction in microfilarial concentration of *D. immitis* in 4 dogs treated with ethanol extract of *Artemisia nilagirica* (Cina θ) at 10 mg/kg/day for 15 days and 20 mg/kg/day for the next 15 days (—○—). The same in 2nd batch of 4 dogs treated with Cina 200 for 30 days (—□—) and in 3rd batch of 4 dogs treated with Cina 1000 for 30 days (—△—).

Percentage changes in microfilarial concentration for the treated dogs were plotted in a graph against days of sampling in Fig. 1. The mean mf density just before treatment of the 3 treatment groups served as the standard with respect to which the percentage change was calculated in subsequent samples. The mf density showed marked reduction in the treated dogs. The maximum reduction was 78.4% with Cina θ, 63.1% with Cina 200 and 71.40% with Cina 1000. The reduction was 42.4-64.2% on the last day of sampling i.e. day 75 (Fig. 1).

Table 2 Spin-lattice relaxation time (T_1) of ^2H of 90% ethanol, Cina θ in 90% ethanol, Cina 200 and Cina 1000. Cina θ is the ethanolic extract of *A. nilagirica* flowering tops. The two potencies, Cina 200 and Cina 1000, were prepared by successive dilution of Cina θ with 90% ethanol 1:100 and manual succussion. Measurements were taken by a AMX -400 NMR spectrometer operating at 61.41 MHz at 22°C

	Mean±S.E. of T_1 (m sec)			
	Water	Ethanol		
	OH	OH	CH_2	CH_3
Ethanol 90%	106.9 ±0.5 a	110.8 ±0.8 a	846.5 ±0.4 a	822.5 ±0.5 a
Cina θ in 90% EtOH	104.3 ±0.4 b	—	883.4 ±0.3 b	776.1 ±0.4 b
Cina 200	108.2 ±0.7 c	102.5 ±0.8 b	867.4 ±1.7 c	792.7 ±0.3 c
Cina 1000	106.6 ±0.8 a	86.8 ±0.9 c	971.7 ±2.4 d	857.6 ±2.3 d

a, b, c, d: Significant difference ($P<0.01$) by ANOVA (one way) in a column.

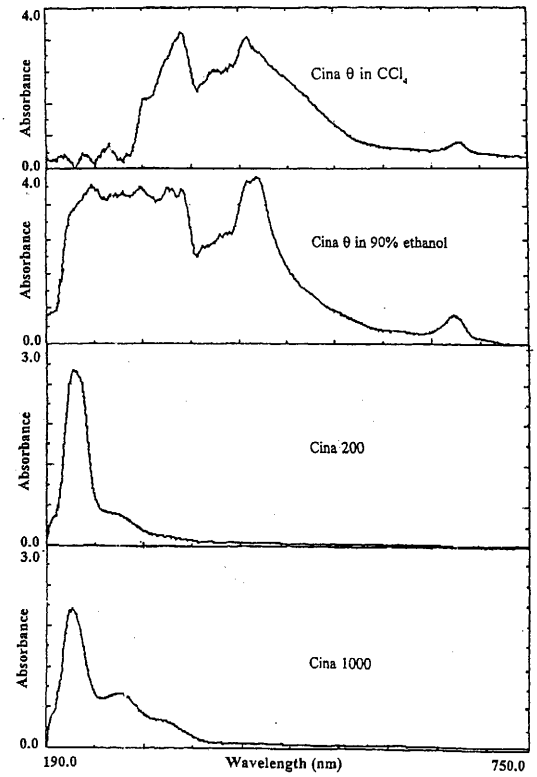

Figure 2 Absorption spectra of Cina θ (ethanol extract of flowering meristems of *A. nilagirica*) and its two ultra high dilutions called Cina 200 and Cina 1000, all in 90% ethanol in wavelength range of 190-750 nm. The same of Cina θ in CCl_4 showing a red shift as compared to Cina θ in 90% ethanol. Cina 200 and Cina 1000 show a blue shift. The absorbance intensities in all the 4 are fairly comparable.

Electronic spectra

Electronic spectra of Cina θ in 90% ethanol, Cina θ in CCl_4, Cina 200 and Cina 1000 are given in Fig. 2. The absorption spectra of Cina θ in ethanol shows a blue shift as compared to that of Cina θ in CCl_4 (Fig. 2). Absorption spectra of Cina 200 and Cina 1000 show a blue shift as compared to Cina θ in ethanol. The absorbance was fairly comparable in the tincture and its two potencies (Fig. 2).

NMR Spectra

The T_1 values with S.E. of ^2H of water, OH, CH_2 and CH_3 of 90% ethanol, Cina θ in 90% ethanol, Cina 200 and Cina 1000 are presented in Table 2. The T_1 values were compared by ANOVA. T_1 of OH of water was lowest with Cina θ and highest with Cina 200. T_1 of ethanol hydroxyl was absent in Cina θ, highest in 90% ethanol and lowest in Cina 1000. T_1 of CH_2 was highest in Cina 1000 followed by Cina θ, Cina 200 and 90% ethanol. T_1 of CH_3 was highest in Cina 1000. This was followed by 90% ethanol, Cina 200 and Cina θ in decreasing order (Table 2).

DISCUSSION

It is evident from the results that both the crude extract as well as the two potencies of *A. nilagirica* proved highly effective against the microfilariae of *Dirofilaria immitis* in dogs. Species of *Artemisia* are reported to contain various types of essential oils and sesqueterpene lactones including santonin (Carnat *et al.*, 1992; Rucker *et al.*, 1992; Nin *et al.*, 1995; Todorrova and Krasteva, 1996). These compounds in the crude extract may have a direct effect on the microfilariae. The effect of the two potencies, which have no drug molecules, can be explained in a different way. Electronic spectra of Cina θ in 90% ethanol and that in CCl_4 are different with the former showing a blue shift as compared to the latter. This suggests molecular complexation and charge transfer (CT) interaction between the compounds of *A. nilagirica* and ethanol molecules. Here ethanol molecules served as electron donors and *Artemisia* compounds as electron acceptors (Singh and Dikshit, 1995). Alcohols donate electrons from the highest occupied nonbonding, n (b_1), orbital of the Oxygen atom to the π orbital of the acceptors in *Artemisia* (Frey *et al.*, 1994). Absorption occurs well into the near UV region. CT is further evidenced by the absence of T_1 value at the ethanolic hydroxyl site of Cina θ (Table 2). Relaxation at this site was too efficient and the n.m.r. signal was too broad to be observed (Banwell and McCash, 1994). We have already observed CT interaction in other potentized homoeopathic drugs like Iodine and Nux vomica (Sukul, 1999). Other plant extracts such as tea contain a mixture of potential complexing agents which form molecular complexes with other compounds (Hernaez *et al.*, 1997). For intermolecular electron-transfer systems, a number of competing acceptors may exist, as in *Artemisia* extract, in a complicated spatial array about the donor. The back transfer process is coupled to the forward transfer in a complex fashion (Weidemaier and Fayer, 1996). Thus the electronic configuration of the donor molecules, i.e. aqueous ethanol, would undergo a change according to the nature of the electronic acceptors of *Artemisia* extract. With successive dilution, the acceptor molecules are progressively depleted and fresh molecules of the donor occupy their place. Finally, it is the molecules of aqueous ethanol which exist in the form of ethanol molecules surrounded by the hydration shell of water molecules.

UV spectroscopy is highly sensitive to the distortion of chromophores and auxochromes (Banwell and McCash, 1994). The UV spectra of Cina 200 and Cina 1000 show a blue shift as compared to that of Cina θ thereby suggesting a possible change in the electronic configuration of the medium, i.e. aqueous ethanol (Fig. 2). The altered T_1 values of the deuterium nuclei of Cina 200 and Cina 1000 as compared to those of aqueous ethanol and Cina θ (Table 2) suggest that the rate of tumbling in the relevant parts of the molecule in potentized Cina has undergone a change obviously due to CT interaction and mechanical agitation (Sukul, 1999). Haseba *et al.* (1993) reported that the thermal motion of water molecules in sonicated aqueous ethanol was greater than that in unsonicated one, and this change in the solution structure produced significant biological effects.

Living microfilariae of *D. immitis,* injected intravenously, disappeared rapidly from the peripheral circulation of uninfected dogs, which had received and cleared previous infections of microfilariae. Eosinophilia and antibodies to microfilaria were demonstrable in the blood of the dogs (Wong, 1964, 1966 cited by Wong and Guest, 1969). Filarial worms are known to cause immunosuppression (Ottesen, 1980). It is possible that potentized Cina might have removed immunosuppression resulting in vigorous responsiveness of the host to parasite antigens thereby clearing microfilariae from the blood.

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DETECTION OF CIRCULATING *WUCHERERIA BANCROFTI* ANTIGEN, FILARIA SPECIFIC IgG AND IgG4 IN CHYLURIA CASES IN JAPAN

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Abstract: Serum samples from Japanese chyluria patients were examined for filaria specific antibodies and a circulating filarial antigen in order to know if the symptom was filarial in origin. All the sera were negative for the circulating antigen. Anti-*Brugia pahangi* antibodies were detected in 6 out of 16 serum samples by ELISA after absorption of the sera with *Anisakis* and *Dirofilaria immitis* antigens. One of the positive sera showed a high titer for anti-*B. pahangi* IgG4, suggesting that *Wuchereria bancrofti* adults were surviving in the patient in recent years. Detection of antibodies would be helpful for immunodiagnosis of filarial chyluria in Japan, where filarial origin is often determined based simply on the history of residence in the past endemic areas.

Key words: *Wuchereria bancrofti,* chyluria, immunodiagnosis, IgG4, circulating antigen

INTRODUCTION

Filariasis caused by *Wuchereria bancrofti* was once a common parasitic disease in Japan, especially in its southern parts. In 1962, the national filariasis control program was launched and an extensive treatment campaign using diethylcarbamazine and mosquito control measures were carried out (Sasa, 1976). By the end of 1970's, microfilaria (mf) carriers were believed to have disappeared in Japan (Kobayashi, 1994). Twenty years after eradication of filariasis, chyluria cases are still encountered (Yagi *et al.,* 1998; Sakakura *et al.,* 1996; Ito *et al.,* 1994; Kawahara *et al.,* 1992; Koyama *et al.,* 1990). In many cases of these reports, the illness was diagnosed or suspected as filarial chyluria simply based on patients' history of residence in the past endemic areas. In this study, a circulating *W. bancrofti* antigen, filaria specific IgG and IgG4 in the sera of chyluria patients were measured in order to obtain immunological evidence for the filarial origin, and to establish an immunological method useful to make a diagnosis of filarial chyluria.

MATERIALS AND METHODS

Serum samples:

Serum samples were obtained in 1994 from 16 chyluria patients and kept at −40°C until use. All the patients were born and brought up in Okinawa Islands, where *W. bancrofti* infection was highly endemic. Information on the patients is shown in Table 1.

Fourteen sera from healthy people living in central parts of Japan were used as healthy controls, and 8 sera from Sri Lankans who were known mf carriers of *W. bancrofti* were used as positive controls.

Detection of *W. bancrofti* circulating antigen:

An enzyme-linked immunosorbent assay (ELISA) for the detection of *W. bancrofti* circulating antigen was carried out using a commercially purchased kit (Trop-Ag *W. bancrofti,* JCU Tropical Biotechnology Pty Ltd., Australia), which is a sandwich ELISA using Og4C3 monoclonal antibody to capture the antigen. The kit has been tested worldwide and its high sensitivity and specificity have been established (More and Copeman, 1990).

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Table 1 Information on the chyluria patients from Okinawa, Japan

Patient No.	Sex	Age	History of filarial infection	Duration of the latest chyluria
1	female	57	—	—
2	male	59	Yes	1 month
3	male	71	—	—
4	—*	—	—	—
5	female	78	—	1 month
6	—	—	—	—
7	—	—	—	—
8	female	60	Yes	2 weeks
9	male	83	Yes	—
10	female	58	—	1 week
11	—	—	—	—
12	female	62	Yes	3 months
13	—	—	—	—
14	male	71	Yes	1 year
15	—	—	—	—
16	male	61	—	1 year

*Information not available.

Antigen preparation for ELISA:

Female adult worms of *Brugia pahangi* collected from the abdominal cavity of Mongolian gerbils, *Dirofilaria immitis* adult worms from the heart of dogs, 3rd-stage larvae of *Anisakis* sp. recovered from mackerels and infective filariform larvae of *Strongyloides ratti* maintained in white rats were homogenized in 1/15 M PBS, pH 7.4, containing proteinase inhibitors: 1 mM of phenylmethane sulfonyl fluoride (PMSF), 10 μM of [L-3-trans-carboxyoxiran-2-carbonyl]-L-leucyl-agmatin (E-64), 0.5 mM of Pepstatin A and 5 mM of ethylenediaminetetraacetic acid (EDTA). Each of the homogenates was centrifuged at 15,000 rpm for 20 min at 4°C and supernatant was obtained. The supernatant was then measured for protein concentration using Bio-Rad Protein Assay Kit (Bio-Rad Lab., USA), adjusted to the required concentration and stored at −40°C until use.

Detection of antibodies to *B. pahangi* with ELISA:

A 96-well microtiter plate was coated with *B. pahangi* antigens (5 μg/ml) at 4°C overnight. The surface of the plate was blocked by casein buffer (1% casein in 0.05 M Tris-HCl buffer with 0.15 M NaCl, pH 7.6) for two hours at room temperature. Serum samples were prepared in five different ways, that is, the sera diluted 400 times with the casein buffer were mixed with the same volume of (1) the buffer itself, (2) the buffer containing *D. immitis* antigens (1 mg/ml), (3) the buffer containing *Anisakis* antigens (1 mg/ml), (4) the buffer containing *S. ratti* antigens (1 mg/ml) or (5) the buffer containing both *D. immitis* and *Anisakis* antigens (1 mg/ml for each antigen). The procedures from (2) to (5) were to absorb antibodies to the corresponding nematodes which may cross-react with the filarial antigens. These mixtures were incubated for 2 hrs at 37°C, then applied to the plate (100 μl/well) and incubated for one hour at 37°C. The plate was washed three times with Tween-PBS (0.05% Tween 20 in 1/15 M phosphate buffered saline, pH 7.4) and affinity purified goat anti-human IgG conjugated with peroxidase (Bio Source International, Inc., Tago Products, USA), which was diluted 4,000 times, was added. After incubation at 37°C for one hour, the plate was washed three times and then ABTS Peroxidase Substrate (KPL, Inc., USA) was added as a substrate for coloration, and absorbance at 415 nm was measured. All antibody levels were expressed as log [absorbance value+1].

For the detection of anti-*B. pahangi* IgG4 antibodies, serum samples were not absorbed as above but simply diluted 500 times. The mouse monoclonal antibody to human IgG4 conjugated with peroxidase (Caltag Lab., Inc., USA) was used as a second antibody.

Results and Discussion

All chyluria cases in this study were regarded clinically as filariasis patients based on the history that they were from the endemic areas. However, there was no concrete evidence to confirm the diagnosis. Many years after eradication of filariasis, it is difficult to differentiate filarial chyluria from that of other etiologies. As adult parasites were reported to survive for up to 17 years, or be reproductive for 5-40 years (Carme *et al.*, 1979), it is still interesting to test if there is any active filarial infection in Japan. For this test, the Og4C3 ELISA which mainly detects *W. bancrofti* adult circulating antigen is applicable. The ELISA has high sensitivity and specificity, and sera collected in the daytime can be used (Lammie *et al.*, 1994). The antigen assay revealed that all the sera from chyluria patients were negative, indicating that there were no surviving parasites in the chyluria patients.

B. pahangi has been a source of antigen for use in serological tests for human lymphatic filariasis (Au *et al.*, 1982; Nuti *et al.*, 1982; Estambale *et al.*, 1994). Immunoglobulin G levels to *B. pahangi* antigens are shown in Figure 1. Without absorption with the nematode antigens (Fig. 1a), IgG levels of mf positive Sri Lankans were clearly higher than those of chyluria patients and healthy controls. Some healthy people

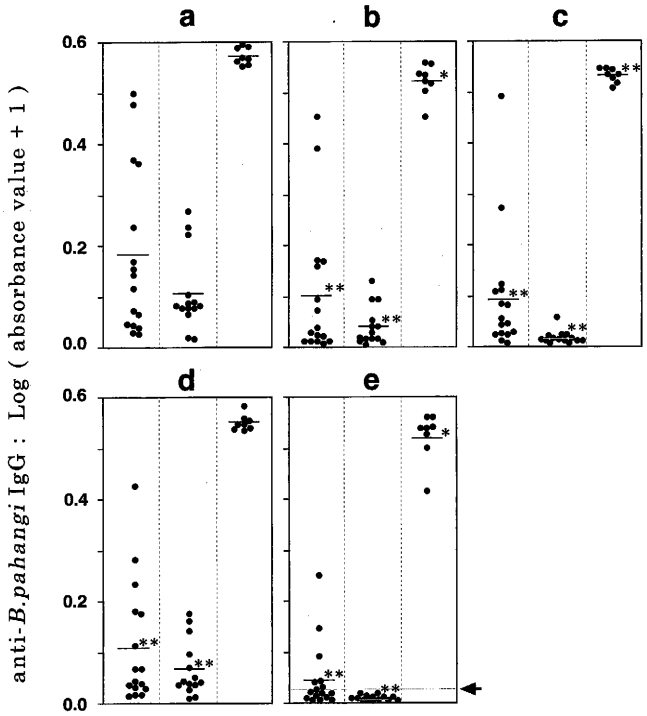

Figure 1 Anti-*B. pahangi* IgG antibodies in serum samples before and after absorption with antigens prepared from *D. immitis, Anisakis* sp. and *S. ratti.*

Note: Serum samples without absorption (a). Samples absorbed with *D. immitis* antigens (b), *Anisakis* antigens (c), *S. ratti* antigens (d), and both *D. immitis* and *Anisakis* antigens (e). The left column of each panel is for chyluria patients, the middle column for Japanese healthy controls and the right column for Sri Lankan mf positives. An arrow in panel (e) indicates a cut-off point. A horizontal bar in each column indicates the mean of antibody titers. The means were compared before and after absorption with *t*-test. Asterisks in panels (b), (c), (d) and (e) indicate levels of significance: *for $p<0.05$, **for $p<0.01$ compared with panel (a).

showed relatively high antibody levels, suggesting that they had been exposed to antigens which produced cross-reactive antibodies to *B. pahangi.* Absorption of these antibodies with *D. immitis, Anisakis* or *S. ratti* antigens significantly reduced absorbance values (Fig. 1b, c, d, e) with one exception: absorption with *S. ratti* antigens did not reduce the values in Sri Lankans (Fig. 1d). The results of statistical tests are shown in Fig. 1. Absorption with *Anisakis* antigens was most effective in reducing absorbance values. Although cross-reactivity of bancroftian filariasis sera with *D. immitis* antigens was well-documented (Grove *et al.,* 1977; Weller *et al.,* 1980), absorption with *D. immitis* antigens was not as effective as *Anisakis* antigens in this study. *S. ratti* was also not effective despite the fact that *Strongyloides stercoralis* is popular in Okinawa (Asato *et al.,* 1992). When cross-reactive antibodies were absorbed with a

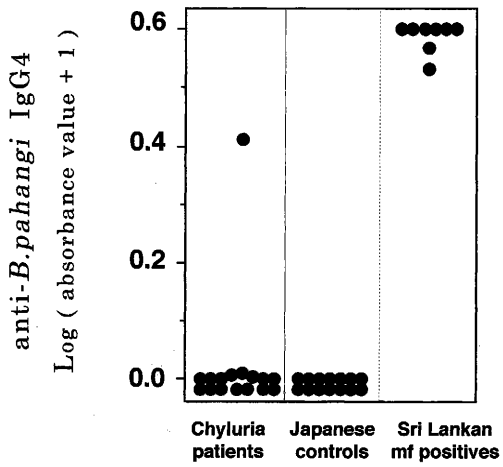

Figure 2 Anti-*B. pahangi* IgG4 antibodies in serum samples.

mixture of *Anisakis* and *D. immitis* antigens, mf positive Sri Lankans, chyluria patients and healthy controls were separated more clearly (Fig. 1e). The absorption by the mixture reduced absorbance values more than the absorption with a single antigen in chyluria patients and healthy controls (*t*-test, $p<0.01$), but not in Sri Lankans. Apparently higher IgG levels to *B. pahangi* in some chyluria patients than in healthy controls (Fig. 1e) would indicate that their clinical sign is filarial in origin.

If the average antibody level of the healthy control + 3SD is regarded as a cut-off point, 6 of 16 chyluria patients could be diagnosed as filarial.

Antibodies of IgG4 subclass to filarial antigens were specifically detected in filariasis patients (Lal and Ottesen, 1988). In addition, the IgG4 antibodies were reported to be associated with active infection of filarial parasites, and disappeared in a relatively short period after treatment (Estambale *et al.,* 1994; Kwan-Lim *et al.,* 1990). In this study, the detection of this subclass resulted in a clear difference between actively infected Sri Lankans and Japanese control subjects (Fig. 2). One chyluria patient, whose sample always showed the highest antibody level before and after absorption, maintained a very high IgG4 level. It is possible some parasites survived until recent years and were stimulating the antibody production. In other words, the detection of IgG4 might be less useful in Japan 20 years after eradication of filariasis.

For immunodiagnosis of filarial chyluria in Japan, there would be two steps to undertake; (1) measurement of *B. pahangi*-reactive IgG 4 and (2) measurement of *B. pahangi*-reactive IgG after absorption of cross-reactive antibodies with *Anisakis* and *D. immitis* antigens. Without having a gold standard of confirmed

filarial chyluria cases, the sensitivity of these immunodiagnoses could not be evaluated. However, in Japan where diagnosis of filarial chyluria is often made without evidence, the present diagnostic method can provide an immunological evidence to support it.

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CLINICAL AND LABORATORY CORRELATES OF THE OUTCOME OF CEREBRAL MALARIA IN THE PHILIPPINES

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Abstract: This study describes the socio-demographic profile of cerebral malaria (CM) patients and identifies the clinical and laboratory factors which correlate with mortality from this condition. Records of 97 CM cases admitted at the Davao Regional Hospital, Philippines from 1990 to 1995 were reviewed. Information on socio-demographic factors, clinical signs and symptoms, physical examination and laboratory findings and medicines given during hospitalization were obtained. Associations of these variables with mortality were examined by generating two-way tables for qualitative variables and computing descriptive statistics for quantitative variables. Results showed that CM was more common among males and young adults who comprise the economically productive age group (21-40 years) and who are engaged in slash and burn agriculture. Using bivariate analysis, factors that correlated with mortality included age, residence, back/abdominal pain, fever, coma, dyspnea, diastolic blood pressure (BP), white blood cell (WBC), neutrophil and lymphocyte counts, trophozoite count and quinine infusion. Logistic regression analysis showed that only back/abdominal pain, coma, dyspnea, diastolic BP and trophozoite count remained statistically significant after simultaneously controlling for confounding. These results can contribute to the proper assessment and improvement in the clinical management of CM.

Keywords: malaria, *Plasmodium falciparum,* cerebral malaria, risk factors, The Philippines

INTRODUCTION

Cerebral malaria is one of the criteria for diagnosis of severe and complicated falciparum malaria. It is defined as unrousable coma which is not attributable to any other cause in a patient with falciparum malaria. The common presentation is that of a generalized convulsion followed by persisting unconsciousness. Among adults, CM is defined as a "non-localizing" motor response to noxious stimuli and an "inappropriate" vocal response. Among children, coma persists for more than half an hour after a convulsion, eye movements are not directed, an "inappropriate cry" is the best verbal response and a non-specific response is the best motor response (WHO, 1990).

CM is the most lethal form of complicated malaria. The mortality and the morbidity from CM remains unacceptably high (Walker *et al.,* 1992). In the Philippines, approximately 31% of the endemic population of about 11 million live in high transmission areas (Malaria Control Service, Department of Health, 1995). Among patients being admitted at the Department of Internal Medicine of Davao Regional Hospital in Tagum, Davao del Norte from the years 1990-1995, malaria was consistently one of the leading causes of morbidity. Malaria mortality in this hospital was usu-

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ally due to its complications, particularly CM. This study was thus conducted to review cases of CM admitted during this 6 year period, establish the socio-demographic profile of CM, describe the clinical manifestations of patients with CM, identify the factors which contribute to mortality and describe the mode of management administered to these patients. The resulting data may serve as an aid to health workers in the prompt recognition of CM as a life threatening condition and guide in the proper assessment of the patient's status, thus improving the diagnosis and management of CM in the Philippines and other malarious countries.

MATERIALS AND METHODS

Subjects

The records of patients, admitted to the Department of Internal Medicine, Davao Regional Hospital, during the period January 1, 1990 to December 31, 1995, and with a diagnosis of CM, were retrieved and reviewed. There were 97 CM cases who satisfied the inclusion criteria -1) final diagnosis of CM and 2) positive malaria smear on admission and on serial examination. The degree of parasitemia was based on the number of parasites counted per field in the thick smear by the following method: (+) if less than 10 parasites were counted per 100 fields, (++) if more than 10 parasites per 100 fields, (+++) if less than 10 parasites per field, and (++++) if more than 10 parasites per field. The socio-demographic data gathered per subject included: age, sex, occupation and place of residence. The prevalence of malaria in each town was noted and the places of residence of the subjects were mapped. The clinical history was reviewed for each subject and pertinent signs and symptoms, and findings on medical and neurologic examination were noted. The fever was classified as: afebrile if temperature$\leqq$37°C, low to moderate$\leqq$38°C and moderate to high grade$>$38°C. Other concomitant conditions such as pregnancy and infections such as pneumonia, tuberculosis, typhoid fever and hepatitis were also noted.

In addition to malaria smear, the other laboratory examinations included in the study were the following: WBC, differential count, blood type and hemoglobin. Other examinations such as fasting blood sugar/random blood sugar, liver function tests, HBsAg, Widal's test, chest x-ray and ECG were done only on few patients and they were not included in the study. The therapeutic regimen given to the patients were also recorded.

Statistical analysis

All the data were encoded using EPI INFO 6 (Version 6.03, CDC, USA; WHO, Geneva). One-way frequency tabulation and descriptive statistics were derived to describe the characteristics of CM patients. To identify characteristics of patients that are related to mortality, the study population was divided into two groups: Group I comprised those subjects who survived (n=53) and Group II were those who died (n=44). The characteristics that were not commonly found among CM patients were not considered for further analysis since the sample size was not enough to draw associations for these variables. For qualitative characteristics, the associations were presented in two-way tables, crosstabulating each factor with mortality status. Odds ratios for all the factors were calculated. Statistical significance of the association between each factor and outcome of disease were assessed using either χ^2 test or Fisher's exact test, whichever is appropriate, at the $\alpha=0.05$ level. For quantitative characteristics, Student's t-test was used to determine statistical significance of the difference of the means between the two groups.

By performing the bivariate analyses described above, several variables were found to be associated with mortality from CM. These variables, however, were also correlated with each other. To remove confounding among these variables, logistic regression analysis was done. Stepwise selection of variables was carried out in arriving at a final model since all variables were considered equally important.

RESULTS

Socio-demographic profile and mortality

Table 1 shows the age and sex distribution of the CM patients admitted in Davao Regional Hospital. The subjects were 13-76 years of age. It was noted that 45.4% belonged to the 21 to 40 age groups. These age

Table 1 Age and sex distribution of CM patients, Davao Regional Hospital, 1990-1995

Age	Male	Female
11-20	10	7
21-30	15	7
31-40	15	7
41-50	11	3
51-60	9	3
61-70	3	3
>71	2	2
Total	65	32

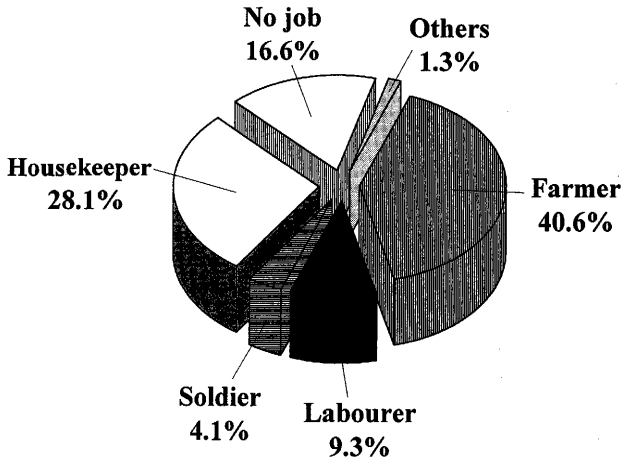

Figure 1 Occupation of the cerebral malaria patients, Davao Regional Hospital, 1990-1995.

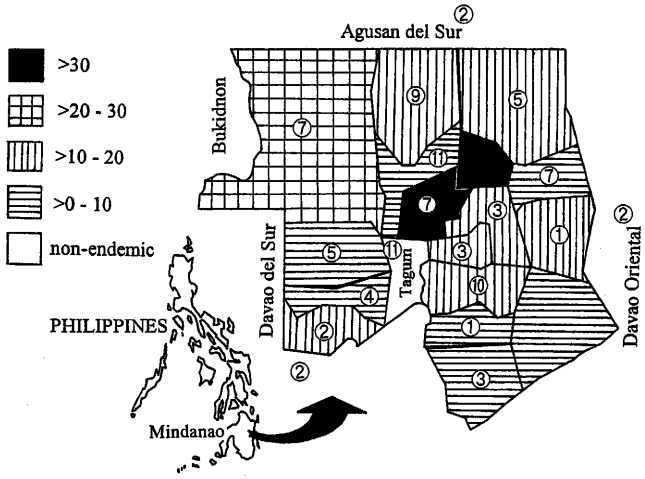

Figure 2 The map of the study areas and their annual parasite incidence per 1,000 in 1993. Numbers in the respective areas are the distribution of cerebral malaria cases.

groups included the adolescents and young adults who comprised the economically productive group of most communities in the Philippines. However, it must be noted that only patients admitted in the Department of Internal Medicine were included as subjects, thus children below 13 years of age were missed in this study. CM had been shown to affect children. A study in another highly endemic area in the Philippines showed the occurrence of CM among children (Yap-delos Santos, 1995). There were more males (67%) than females (33%). This may be due to the greater malaria exposure of the male population because of occupation. Farmers, engaged in slash and burn agriculture in forested areas, comprised 40.6%, while 28.1% were housekeepers who were exposed to malaria by residing in endemic foci. Laborers comprised 9.3%, soldiers 4.1% and 16.6% had no job (Figure 1). Figure 2 shows the geographic distribution of the residence of the CM

Table 2 Odds Ratio and 95% confidence intervals for the association of socio-demographic characteristics with outcome of CM

Socio-demographic characteristics	Died	Recovered	Odds Ratio[a]	95% confidence interval	p-value
Sex					
Male	28	37	0.76	0.30-1.93	0.5196
Female	16	16			
Age					
11-20	5	12	1.00	—	
21-30	9	13	1.66	0.36-7.92	0.4580
31-40	15	7	5.14	1.08-26.34	0.0163
41-50	6	8	1.80	0.32-10.37	0.4362
51-above	9	13	1.66	0.6-7.92	0.4580
Occupation					
Exposed[b]	27	26	1.65	0.68-4.03	0.2255
Not exposed[c]	17	27			
Residence					
Non-endemic	10	5	1.00	—	—
>0-10 cases/1,000 pop'n	14	17	0.41	0.09-1.75	0.1711
>10-20 cases/1,000 pop'n	17	20	0.43	0.10-1.73	0.1755
>20 cases/1,000 pop'n	3	11	0.14	0.02-0.92	0.0144

[a]whenever there are more than 2 categories, the first is treated as the reference group
[b]farmers, laborers, soldiers
[c]housekeepers, no job

Table 3 Odds Ratios and 95% confidence interval for the association of clinical findings with outcome of CM

Signs and Symptoms	Died	Recovered	Odds Ratio	95% Confidence Interval	p-value
Headache					
+	16	24	0.69	0.28-1.69	0.3743
−	28	29			
Malaise					
+	8	11	0.85	0.27-2.61	0.7506
−	36	42			
Fatigue					
+	3	1	3.80	0.29-203.51	0.2418
−	41	52			
Dizziness					
+	1	0	—	—	0.4536
−	43	53			
Back/Abdominal Pain					
+	7	1	9.84	1.16-451.68	0.0153
−	37	52			
Anorexia					
+	11	6	2.61	0.78-9.42	0.0777
−	33	47			
Vomiting					
+	3	1	3.80	0.29-203.51	0.2418
−	41	52			
Diarrhea					
+	1	1	1.20	0.02-96.73	0.7040
−	43	52			
Chills					
+	22	37	0.43	0.17-1.08	0.0466
−	22	16			
Fever					
none	24	44	1.00		
low-moderate	5	3	3.06	0.53-21.06	0.1335
moderate-high	15	6	4.58	1.42-16.12	0.0035
Prostration					
+	4	0	—	—	0.0392
−	40	53			
Conscious					
+	18	26	0.72	0.32-1.74	0.4223
−	26	27			
Coherent					
+	7	9	0.92	0.27-3.11	0.8874
−	37	44			
Incoherent					
+	3	8	0.41	0.07-1.88	0.1696
−	41	45			
Unresponsive					
+	17	12	2.15	0.81-5.76	0.0867
−	27	41			
Lethargic					
+	15	9	2.53	0.89-7.43	0.0519
−	29	44			
Stuporous					
+	17	14	1.75	0.68-4.55	0.1988
−	27	39			

Comatose					
+	13	3	6.99	1.76-40.46	0.0016
−	31	50			
Dyspnea					
+	20	1	43.33	6.01-1829.33	0.0000
−	24	52			
Focal seizures					
+	5	0	—	—	0.0169
−	39	53			
Neck rigidity					
+	1	1	1.21	0.02-96.73	0.7040
−	43	52			
Visual disturbances					
+	4	1	5.20	0.48-260.90	0.1285
−	40	52			
Abdominal reflexes					
+	3	3	1.22	0.15-9.58	0.5687
−	41	50			
Liver dysfunction					
+	15	13	1.59	0.60-4.24	0.3008
−	29	40			
Anaemia and bleeding					
+	11	11	1.27	0.44-3.68	0.6191
−	33	42			
Shock					
+	1	0	—	—	0.4536
−	43	53			
Blackwater fever					
+	1	0	—	—	0.4536
−	43	53			
Acute renal failure					
+	3	0	—	—	0.0898
−	41	53			
Pulmonary edema					
+	11	5	3.20	0.91-12.73	0.0397
−	33	48			
Hepatitis					
+	1	0	—	—	0.4536
−	43	53			
Pulmonary tuberculosis					
+	0	1	—	—	0.5464
−	44	52			
Pneumonia					
+	5	3	2.14	0.39-14.49	0.2586
−	39	50			

subjects. All these areas except Tagum were known to be endemic with malaria prevalence ranging from 0.2 to 47.9 per 1,000 in 1993 (Malaria Control Service, Davao del Norte). A few (11.3%) of CM subjects resided in non-endemic, urban Tagum.

Table 2 shows the association of selected demographic characteristics with mortality. For this analysis, occupations were classified as "exposed" and "unexposed" to malaria while residence were categorized according to prevalence of infection in the area. Age and residence of the patient were found to be significantly associated with outcome of CM while sex and occupation were not. Mortality was significantly higher among the 31-40 year age group compared to those below 21 years ($p=0.0163$). Patients from non-endemic areas also had significantly higher mortality compared to patients of highly endemic areas ($p=0.0144$).

Clinical manifestations, physical findings and mortality

Table 3 shows the Odds Ratios and p-values for determining the association between the outcome of malaria and the presence of certain signs and symptoms, physical examination findings, other complications of malaria and concomitant infections. Only back/abdominal pain, fever, dyspnea and coma, were shown to be associated with a fatal outcome of CM ($p<0.05$). None of the subjects complained of chest pain, nausea and sweating. There were no subjects who were oriented, or presented with generalized convulsions, poor muscle tone and ankle clonus. None of the subjects had reactive psychosis, delirium, cerebellar disturbances, hypoglycemia and neurologic sequelae. Table 4 of the vital signs shows that only a higher diastolic BP was associated with fatal outcome of CM ($p<0.05$).

Laboratory findings and mortality

Table 5 shows the association of the laboratory findings with the outcome of CM. The WBC, neutrophil and lymphocyte counts showed statistical significance ($p<0.05$). Table 6 of the blood type shows that there was no statistically significant association between blood type and the outcome of CM ($p<0.05$). Table 7 shows that trophozoite count (+++/++++) was statistically associated with poor outcome of CM ($p<0.05$).

Antimalarial therapy and supportive management

The only available regimen for complicated and severe malaria in the Philippines is intravenous Quinine infusion of 10 mg/kg in 500 m*l* of 5% dextrose in water, 125 m*l* of which was given in a drip for 4 hr at 6 hr intervals. In 85.5% of subjects, the infusion was started on admission and 14.4% on the succeeding hospital days. The latter subjects were conscious on admission and so, they were given oral antimalarials such as Chloroquine phosphate tablets, 10 mg base/kg initially, then 5 mg base/kg after 6 hr, then 5 mg base/kg on days 2 and 3; Sulfadoxine (25 mg/kg), Pyrimethamine (1.25 mg/kg) tablets, single dose. Deterioration of the level of consciousness led to the shifting from oral to intravenous Quinine. Table 8 shows the association of medicine given with the outcome of CM. Quinine infusion correlated with a good outcome of CM ($p<0.05$).

Stepwise logistic regression analysis

Several signs and symptoms had statistically significant associations with mortality from CM. Since these variables may be mutually confounding each other,

Table 4 Mean and standard deviation (S.D.) of vital signs by outcome of CM

Vital sign	Died			Recovered			Test statistics (p-value)
	Sample Size	Mean	S.D.	Sample Size	Mean	S.D.	
Systolic blood pressure (mmHg)	42	110.0	21.9	52	103.5	13.8	1.76 (0.0811)
Diastolic blood pressure (mmHg)	42	72.6	13.3	52	66.7	11.8	2.27 (0.0254)
Temperature (°C)	44	38.4	1.2	52	38.6	1.1	0.57 (0.5702)
Heart rate (per minute)	39	89.2	22.9	50	91.8	15.8	0.63 (0.5294)

Table 5 Mean and standard deviation (S.D.) of laboratory findings by outcome of CM

Laboratory findings	Died			Recovered			Test statistics (p-value)
	Sample Size	Mean	S.D.	Sample Size	Mean	S.D.	
Hemoglobin (g/L)	43	101.9	32.0	53	97.4	27.8	0.74 (0.4627)
WBC ($\times 10^9$/L)	43	12.6	8.85	51	8.1	4.30	3.21 (0.0018)
Neutrophil count	43	0.74	0.10	51	0.68	0.11	2.52 (0.0134)
Lymphocyte count	43	0.26	0.10	51	0.32	0.11	2.51 (0.0138)

Table 6 Odds Ratios and 95% confidence intervals for the association of blood type with the outcome of CM

Blood type	Died	Recovered	Odds Ratio	95% confidence interval	p-value
A	10	14	1.00	—	
AB	2	3	0.93	0.07-9.84	0.6705
B	13	11	1.65	0.46-6.09	0.3861
O	15	18	1.17	0.35-3.87	0.7760

simultaneously controlling for this effect had to be done by logistic regression analysis. To prevent missing clinically important variables, associations of variables with mortality that had a p-value≦0.15 were considered in the analysis. Prostration, focal seizures and acute renal failure had zero frequency in one cell and were not considered for further analysis. Stepwise selection of variables was performed to determine which variables remained significant ($\alpha=0.10$) after controlling for confounders. Age and residence of subjects were considered confounders and were forced in into the model.

The results of logistic regression analysis are shown in Table 9. The variables that entered the model were presence of back/abdominal pain, coma, dyspnea diastolic BP (>90 mm Hg) and trophozoite count. This means that the association of these variables with mortality from CM are less likely to be due to confounding.

Table 7 Odds Ratios and 95% confidence intervals for the association of parasite count with outcome of CM

Parasite count	Died	Recovered	Odds Ratio	95% confidence interval	p-value
P. falciparum trophozoites					
+	13	25	1.00		
++	9	17	1.02	0.31-3.30	0.9733
+++/++++	22	11	3.85	1.29-11.73	0.0064

\+ =if less than 10 parasites were counted per 100 fields
++ =if more than 10 parasites per 100 fields
+++ =if less than 10 parasites per field
++++=if more than 10 parasites per field

Table 8 Odds Ratios and 95% confidence intervals for the association of medicines given with outcome of CM

Medicine given	Died	Recovered	Odds Ratio	95% confidence interval	p-value
Quinine					
+	13	41	0.12	0.04-0.33	0.0000
−	31	12			
Chloroquine					
+	2	5	0.46	0.04-3.00	0.3020
−	42	48			
Mannitol					
+	7	11	0.72	0.21-2.30	0.5411
−	37	42			
Fansidar					
+	0	2	—	—	0.2960
−	44	51			

Chloroquine phosphate 10 mg base/kg initially, then 5 mg base/kg after 6 hr, then 5 mg base/kg on days 2 and 3.
Sulfadoxine (25 mg/kg)-pyrimethamine (1.25 mg/kg) single dose.
Quinine by intravenous infusion 10 mg/kg in 5% dextrose over 4 hr.
Mannitol by intravenous infusion 1 g/kg of 10% solution over 30 min.

Table 9 Results of stepwise logistic regression analysis

Variable	Parameter estimate	Standard error	p-value	Odds Ratio
Back/abdominal pain	2.6275	1.4619	0.0723	13.840
Coma	2.5548	0.9549	0.0075	12.868
Dyspnea	3.6037	1.2432	0.0037	36.732
Diastolic blood pressure	2.7273	1.2859	0.0339	15.292
Trophozoite count (++)	1.0665	0.8396	0.2040	2.905
Trophozoite count (+++/++++)	1.7802	0.8181	0.0296	5.931

DISCUSSION

From 1946 to 1952, the Department of Health reported that malaria was the 5th cause of mortality in the Philippines. Due to control/eradication efforts, the mortality rate went down and, at present, it is only 1.5 per 100,000 population (Asinas, 1993; Malaria Control Service, Manila, 1995). However, in some areas in the Philippines, malaria is still a leading cause of death and, for example in a highly endemic area, the mortality rate from CM was 19% in children (Yap-delos Santos, 1995). In two other studies, reported mortality rates among CM cases varied from 20% (Alcantara *et al.*, 1982) to 35% (Tagle and Cabanban, 1992). In our present study which was done in another highly endemic area, the mortality rate of CM among adults was 45%. Such high mortality in CM and the factors which correlated with a bad outcome or death, were the focus of this study.

The socio-demographic profile of CM patients in our study showed that almost half belonged to the age group who are most economically productive and active members of the community. Males predominated, suggesting that malaria exposure may be related to occupation. It was observed that most CM patients were farmers engaged in slash and burn agriculture and residing in endemic areas. However, occupation and sex did not show statistically significant association with mortality from CM. Instead, mortality was statistically associated with 31-40 years age group and a place of residence which is none to low endemic for malaria. This may be due to non-suspecting health personnel resulting in delay of CM diagnosis and treatment.

Alcantara (1982), Tagle and Cabanban (1992), described that the signs and symptoms commonly observed in the fatal cases of CM included coma, oliguria, jaundice, hyperpyrexia, headache, and chills. In our study, back/abdominal pain, fever, dyspnea, coma, diastolic BP, WBC, neutrophil and lymphocyte counts and trophozoite count correlated with a poor outcome ($p<0.05$). Quinine infusion on the other hand correlated with good outcome ($p<0.05$). The presence of coma has always been the risk factor for grave prognostic outcome in CM (WHO, 1990; Walker *et al.*, 1992). Such finding was shown in this study. For the other physical examination findings, it was shown that a higher diastolic BP correlated with poor outcome. Those CM patients who died exhibited a higher reading compared to those who recovered from CM (Table 4).

With regards to the laboratory findings, the total WBC, neutrophil and lymphocyte counts correlated with poor outcome ($p<0.05$). Those CM patients who died had higher total WBC and neutrophil counts and lower lymphocyte count. This may be a significant finding particularly in relation to the recent finding of tissue infiltration by leukocytes in autopsy specimens of young adults with CM in Orissa State, India (Mendis and Carter, 1995). Peripheral leukocytosis ($>12{,}000/\mu l$) is a laboratory index of poor prognosis in CM (WHO, 1990). An interesting finding was that the mean hemoglobin levels (Table 5) were not so low as reported in the study of children where 21% had less than 7.0 g/l (Yap-delos Santos, 1995). In this study, the mean hemoglobin of those who died was 10.1 g/l. Severe anemia may be more common in children as in Africa, where it is a common feature of complicated disease and causes the most mortality during the first two years of life (Greenwood *et al.*, 1987; Mendis and Carter, 1995). However, adults may not be that susceptible to anemia as children. As for blood-group antigens, they have been implicated among the ligands of rosetting on infected erythrocytes, which causes obstruction of cerebral blood flow in CM. There is some evidence to suggest that severe malaria may be relatively less frequent among O blood type because unlike A and B erythrocytes, O erythrocytes do not exhibit rosetting (Mendis and Carter, 1995). However, this study did not demonstrate a significant correlation between blood types and a poor outcome of CM (Table 6).

It has been shown that hyperparasitemia (>5% parasitized erythrocytes or >250,000 parasites per μl) is a laboratory index of poor prognosis in CM (WHO, 1990). However, one study did not show any difference between parasite count in the fatal and non-fatal cases (Walker *et al.*, 1992). In our study, a semi-quantitative count of +++/++++ (one or more parasites per 1 thick field) correlated with a poor outcome of CM ($p<0.05$) (Table 7).

Walker *et al.* (1992), WHO (1990), showed that hypoglycemia is common and is associated with a poor outcome. In this study, glucose determinations were not done in many patients and analysis for its association with the outcome could not be done.

The management of CM is straightforward as long as the diagnosis is made. Quinine is a very effective drug in complicated malaria including CM. However, failure to reduce mortality may be due to lack of supportive management. Anticipatory, prompt treatment, and supportive management for a life-threatening condition such as CM, may be the key in reducing the mortality.

This study used data from medical records before it was conducted. It is very likely that some conditions might have been present in some patients but were not

recorded if the physician did not think these were critical in the management of the patient. Unfortunately, the effect of this misclassification cannot be assessed in this study.

From the results of logistic regression analysis, presence of back/abdominal pain, coma, dyspnea, diastolic BP (>90 mm Hg) and trophozoite count were significant. These variables were found to be significant after controlling for confounders. The odds ratios, however, cannot be interpreted reliably due to small sample size of the study. This may also account for the exclusion of other important variables from the model. A larger study would confirm these associations and estimate the effect of these variables on mortality more precisely.

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FOUR NEW SPECIES OF BLACK FLIES (DIPTERA: SIMULIIDAE) FROM THAILAND

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Abstract: Four new species of black flies (Diptera: Simuliidae) are described based on reared adult, pupal and/or larval specimens collected from Thailand. Three new species, *Simulium chaliowae* sp. nov., *S. chainarongi* sp. nov., and *S. triglobus* sp. nov., are all assigned in the *multistriatum*-group of the subgenus *Simulium* (*Simulium*), and share the similar shoe-shaped cocoon instead of the usual fenestrate cocoon. *S. triglobus* is most remarkable by having three spermathecae in female adult. The fourth new species, *S. baimaii* sp. nov., is placed in the genus *Simulium* but its subgeneric assignment remains unclear due to lack of adult specimens. This species is distinct from the other known simuliid species in the Oriental Region by having the pupal gill with two filaments directed forward from an inflated stalk on each side.

Key words: *Simulium,* black fly, Simuliidae, Thailand, spermatheca, new species

Takaoka and Suzuki (1984) reported 19 species of black flies (Diptera: Simuliidae) from Thailand, including seven new species and five unnamed ones. Takaoka and Saito (1996) described a new species and recorded four other simuliid species for the first time from this country. In the same paper, three species, previously reported as *Simulium sakishimaense* Takaoka, *S. nitidithorax* Puri, and *S.* sp. C, were identified as *S. fenestratum* Edwards, *S. tani* Takaoka and Davies, and *S. asakoae* Takaoka and Davies, respectively. Takaoka and Adler (1997) reported two more species from Thailand. In total, 26 black-fly species including four unnamed species have been known from Thailand.

Recently, one of us (CK) made a faunistic survey of Simuliidae in northern and northeastern provinces of Thailand, and collected four new species, of which three are assigned in the *multistriatum*-group of the subgenus *Simulium* (*Simulium*), redefined by Takaoka and Davies (1996). All these three are characterized by the shoe-shaped cocoon with an anterior collar of variable heights, in place of the usual fenestrate cocoon. Strikingly, one of these species has three spermathecae in the female adult. The fourth new species, represented only by the pupal and larval stages, is also placed in the same genus but remains unclassified into any known subgenus. This species is also very distinctive in having the pupal gill with two filaments, a character very rarely encountered in the Simuliidae.

The morphological features and terms used herein follow mostly those of Crosskey (1969), and partially those of Takaoka (1983). All holotype and some paratype specimens will be deposited in the Department of Biology, Faculty of Science, Mahidol University, Bangkok, Thailand, and some other paratypes, in the Department of Entomology, The Natural History Museum, London, UK.

Simulium (*Simulium*) *chaliowae* Takaoka and Boonkemtong sp. nov.

Female. Body length 2.2 mm. ***Head.*** Narrower than thorax. Frons black, shiny, with several dark stout hairs along lateral margins; frontal ratio (width: height—width greatest near vertex, narrowest near antennal base) 1.2:1.0:1.6; frons-head ratio (greatest width of frons: greatest width of head) 1.0:4.5. Fronto-ocular area (Fig. 1) well developed. Clypeus black, shiny, gray-pruinose, iridescent when illuminated, moderately covered with dark stout hairs. Antenna composed of 2+9 segments, yellow on scape, pedicel and most of 1st flagellar segment, and brownish on rest of flagella, when viewed dorsally; while antenna yellow on scape, pedicel, and a few flagellar segments, and gradually darkened toward apex on rest of flagella, when viewed anteriorly or ventrally. Maxillary palp with 5 segments, light to dark brown, proportional lengths of

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3rd, 4th and 5th segments 1.0:1.2:2.2; 3rd segment (Fig. 2) of normal size, with sensory vesicle oblong, ca. 0.4× as long as 3rd segment. Maxillary lacinia with 12 or 13 inner teeth and 12 outer teeth. Mandible with ca. 22 inner teeth and ca. 14 outer teeth. Cibarium with a blunt, medial projection on posterior margin, and without any tubercles, similar to that of *S. chainarongi* sp. nov. ♀ (Fig. 24). ***Thorax***. Scutum brownish black, shiny, densely covered with whitish-yellow, recumbent pubescence, and with several dark upright hairs on prescutellar area; when illuminated in front and viewed dorsally, scutum thickly white-pruinose, with 5 longitudinal unpruinose vittae, of which 1 median vitta narrowest, 2 sublateral ones widest, and 2 submedian ones intermediate, slightly or somewhat wider than median one but much narrower than sublateral ones, all vittae united with broad transverse band on prescutellar area; when illuminated from behind, scutum having reversed color pattern. Scutellum light brown, covered with dark upright hairs as well as whitish-yellow pubescence. Postscutellum brownish black, shiny, gray-pruinose, bare. Pleural membrane bare. Katepisternum longer than deep, bare. ***Legs***. Foreleg: coxa and trochanter yellowish white; femur yellow on basal 2/5 or a little more, and dark brown on the rest when viewed outwardly (border well defined), while yellowish portion extending more apically beyond basal 1/2 when viewed inwardly (border not well defined); tibia white to yellowish white except apical 1/6 or 1/5 brownish black, with large area of white sheen on outer surface; tarsus brownish black; basitarsus somewhat dilated, ca. 4.8× as long as its greatest width. Midleg: coxa blackish brown, trochanter light brown except basal 1/2 yellow; femur blackish brown except basal 1/5 or 1/4 yellow; tibia yellowish white except apical 1/6 blackish brown, with large area of white sheen on posterior surface; tarsus blackish brown except basal 2/3 of basitarsus yellowish. Hind leg: coxa blackish brown; trochanter whitish yellow; femur blackish brown except basal 1/5 or 1/4 whitish yellow; tibia white with apical 1/3 or 1/4 blackish brown, with large area of white sheen on posterior surface; tarsus blackish brown except basal 3/5 of basitarsus and basal 1/2 of 2nd segment whitish yellow; basitarsus (Fig. 4) parallel-sided; calcipala moderately developed, as long as wide or slightly shorter than wide, and pedisulcus well developed at basal 1/3 of 2nd tarsal segment. All tarsal claws simple. ***Wing***. Length ca. 2.2 mm. Costa with spinules and hairs; subcosta haired except near apex bare; basal section of radial vein bare except near apex where 1-4 hairs are present; hair tuft on stem vein brown. ***Abdomen***. Basal scale light yellowish brown, with fringe of pale long hairs, of which basal portions are dark. Dorsal surface of 2nd abdominal segment pale yellowish white with narrow area just before posterior margin brownish black; dorsal surface of remaining segments brownish black, with short dark hairs; terga 6-8 shiny. Ventral surface of 7th segment with a pair of small submedial sternal plates. ***Genitalia*** (Figs. 6-8). Sternite 8 moderately sclerotized, bare in middle, covered with 30-34 stout hairs (in another female, 23-25 hairs) on each lateral surface. Anterior gonapophyses triangular, moderately separated from each other medially, membraneous, covered with numerous microsetae and 1-3 stout hairs; narrow bare area along inside and posterior margins very thin and transparent (then often overlooked). Genital fork of inverted-Y form, with narrow, well sclerotized stem; arms of moderate width, each with distinct projection directed anteriorly. Paraproct much protruding ventrally, ca. 1.4× as wide as long, with numerous stout hairs on lateral and ventral surfaces. Cercus short, ca. 1.7× as wide as long, with numerous stout hairs, with rounded posterior margin, when viewed laterally. Spermatheca 1 in number, nearly globular (in another female, spermatheca is also globular but somewhat smaller as shown in Fig. 8), well sclerotized except tubal base widely unsclerotized, and with internal setae; spermathecal duct with 2 additional ducts at base, which are equally short, ca. 0.3× as long as main duct, but with no apical spermathecae.

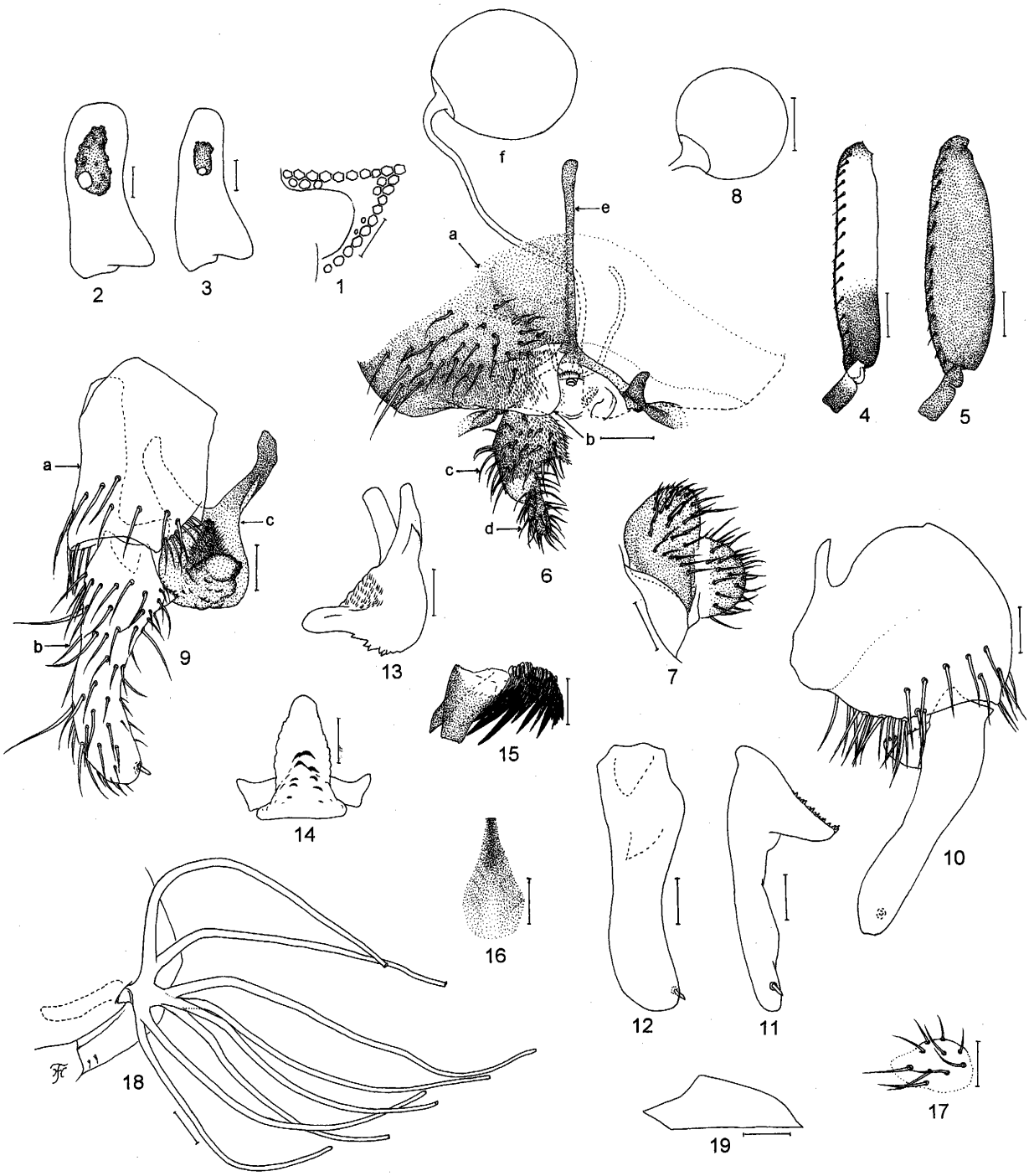
Male. Body length ca. 2.6 mm. ***Head***. Slightly wider than thorax. Upper eye consisting of 17 vertical columns and 18 horizontal rows of large facets. Face and clypeus black, white-pruinose, silvery iridescent when illuminated, sparsely covered with long dark hairs. Antenna composed of 2+9 segments, dark brown except scape, pedicel and base of 1st flagellar segment dark yellow; 1st flagellar segment somewhat elongate, ca. 1.6× as long as 2nd flagellar segment. Maxillary palp with 5 segments, dark brown to blackish brown, proportional lengths of 3rd, 4th and 5th segments 1.0:1.3:2.5; sensory vesicle (Fig. 3) small, oblong, ca. 0.2× as long as 3rd segment. ***Thorax***. Scutum black, densely covered with golden yellow, recumbent pubescence, as well as a few brown upright hairs on prescutellar area; scutum in certain angles of light with silvery iridescent spots, i.e., an anterolateral pair of spots on shoulders which connect widely to posterior large spot on prescutellar area through spots along lateral margins; an anterolateral pair of spots large, widely separated in middle from each other. Scutellum brownish black, with

golden yellow pubescence as well as dark upright hairs. Postscutellum brownish black, silvery iridescent when illuminated, and bare. Pleural membrane and katepisternum as in ♀. ***Legs***. Foreleg: coxa yellow; trochanter light brown; femur light to dark brown, with base somewhat paler; tibia light to dark brown with large median area on outer surface white, and shining when illuminated; tarsus brownish black; basitarsus somewhat dilated, ca. 5.8× as long as its greatest width. Midleg: coxa brownish black; trochanter and femur blackish brown; tibia whitish yellow with apical 2/5 blackish brown (its border not well defined); tarsus blackish brown except basal 1/2 of basitarsus dark yellow (border not well defined). Hind leg: coxa brownish black; trochanter yellow; femur and tibia light brown with base yellow and apical cap dark brown; tarsus light brown; basitarsus (Fig. 5) enlarged, ca. 3.6× as long as its greatest width, and subequal in greatest width to hind tibia; calcipala and pedisulcus well developed. ***Wing***. Length ca. 2.0 mm; other features as in ♀ except subcosta hairy with apical 1/4 bare. ***Abdomen***. Basal scale light brown medially, dark brown laterally, with fringe of dark long hairs. Dorsal surface of abdominal segments brownish black except basal 1/2 of 2nd segment yellowish brown, with short dark hairs; terga 2, 5, 6 and 7 each with a pair of dorsolateral silvery spots, those on tergum 2 connected broadly to each other medially. ***Genitalia*** (Figs. 9-17). Coxite subquadrate in ventral view, with stout hairs only near posterior margin. Style elongate, much longer than coxite, gently curved inwards, moderately covered with stout hairs on ventral and lateral surfaces, and with single subterminal spine; style widest subbasally, hence narrowed toward midpoint, then slightly widened toward apical 1/4, thereafter parallel-sided up to apical 1/8, and ca. 3.4× as long as its greatest width, which is ca. 1.6× the narrowest width at middle; style with distinct basal protuberance directed dorsomedially, with several small cone-shaped spines along anterior margin and at apex. Ventral plate in ventral view subquadrate (though its posterolateral corners rounded), somewhat longer than wide, with prominent median process directed ventrally, of which apex is rounded and bare, posterior surface has ca. 10 teeth in 2 vertical irregular rows, and anteroventral surface is covered with microsetae medially; basal arms strongly sclerotized, and divergent from each other. Paramere with several hooks. Median sclerite thin, widened toward apical tip. Cercus small, short, with several hairs.

Pupa. Body length ca. 2.8 mm. ***Head*** and ***Thorax***. Integument yellow, densely and elaborately covered with round tubercles (cone-shaped tubercles on posterodorsal surface) of different sizes; head with 1 facial and 2 frontal pairs of simple trichomes, facial trichomes medium in length and somewhat longer than frontal ones; thorax on each side with 3 long trichomes each with 3-4 branches mediodorsally, 1 bifid medium and 1 simple long trichomes anterolaterally, 1 simple long trichome posterolaterally and 3 simple or bifid trichomes (2 long and 1 medium) ventrolaterally, all long trichomes rather stout. Gill (Fig. 18) with 8 short, slender filaments in pairs (all short-stalked), diverging widely not only vertically but also horizontally, with an angle formed at base by dorsalmost and ventralmost filaments nearly 170 degrees; filaments somewhat decreasing in length (from ca. 1.2 mm to 0.8 mm) and thickness (at least basal portion) from dorsal pair to ventral one; basal portion of dorsalmost filament ca. 1.4× as thick as ventralmost filament, and slightly thinner than interspiracular trunk; 2 filaments of all pairs subequal in thickness to each other; all filaments light yellow, tapered toward apex, with distinct annular ridges and furrows forming elaborate reticulate surface patterns, and densely covered with minute tubercles. ***Abdomen***. Tergum 1 light brown, bare or sparsely tuberculate, with 1 simple or bifid medium seta on each side; tergum 2 pale, with 1 simple medium seta and 5 short spines (of which 4 are much stout) on each side; terga 3 and 4 each with 4 hooked spines and 1 simple short spinous seta, on each side; tergum 8 with distinct spine-combs in transverse row, on each side; terga 7 and 9 each with or without a few, much smaller spine-combs; terga 5 and 6 bare; terminal hooks absent. 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 outer hooks widely spaced, on each side. ***Cocoon*** (Fig. 19). Shoe-shaped, with anterior collar of moderate height, thinly woven; there appear to be some open spaces in webs of anterior collar, but it is difficult to ascertain this because the entire surface of the cocoons was thickly cemented by silicic material.

Mature larva. Unknown.

TYPE SPECIMENS. Holotype ♀, reared from pupa, collected from Maekham waterfall, Phare Province, northern Thailand, 10. XII. 1998, by Chaliow Kuvangkadilok and Chainarong Boonkemtong. Paratype 1 ♀, 1 ♂, reared from pupa, 1 pharate ♀ and 1 pharate ♂, same data as holotype.



Figures 1-19 Morphological characters of *S.* (*S.*) *chaliowae* sp. nov. 1, ♀ fronto-ocular area; 2 and 3, 3rd segments of maxillary palp with sensory vesicle inside (2, ♀; 3, ♂); 4 and 5, basitarsi and second tarsal segments of hind legs showing calcipala and pedisulcus (4, ♀; 5, ♂); 6, ♀ genitalia *in situ* (ventral view; left paraproct and cercus omitted) showing 8th sternite (a), anterior gonapophysis (b), right paraproct (c), right cercus (d), genital fork (e), and spermatheca (f); 7, right paraproct and cercus (lateral view); 8, spermatheca of paratype ♀; 9, ♂ genitalia *in situ* (ventral view; left coxite, style, and parameres omitted) showing right coxite (a), right style (b) and ventral plate (c); 10, right coxite and style (lateral view); 11, right style (medial view) showing basal protuberance with spines along anterior margin; 12, right style (ventrolateral view); 13 and 14, ventral plates (13, lateral view; 14, end view); 15, paramere (end view); 16, median sclerite (end view); 17, left cercus (lateral view); 18, pupal gill filaments and interspiracular trunk (lateral view); 19, cocoon (lateral view). Scale bars 1.0 mm for fig. 19; 0.1 mm for figs. 4, 5, and 18; 0.05 mm for figs. 1, 6, 7 and 8; 0.03 mm for figs. 9-17; 0.02 mm for figs. 2 and 3.

ECOLOGICAL NOTES. This species was found on fallen leaves in a fast flowing stream 3 m wide at Maekham waterfall (290 m in altitude), where the water temperature was 20°C. This species was found together with *S. fenestratum, S. quinquestriatum, S. nakhonense, S. tani* and *S. siamense.*

ETYMOLOGY. The species *chaliowae* is named after Dr. Chaliow Kuvangkadilok.

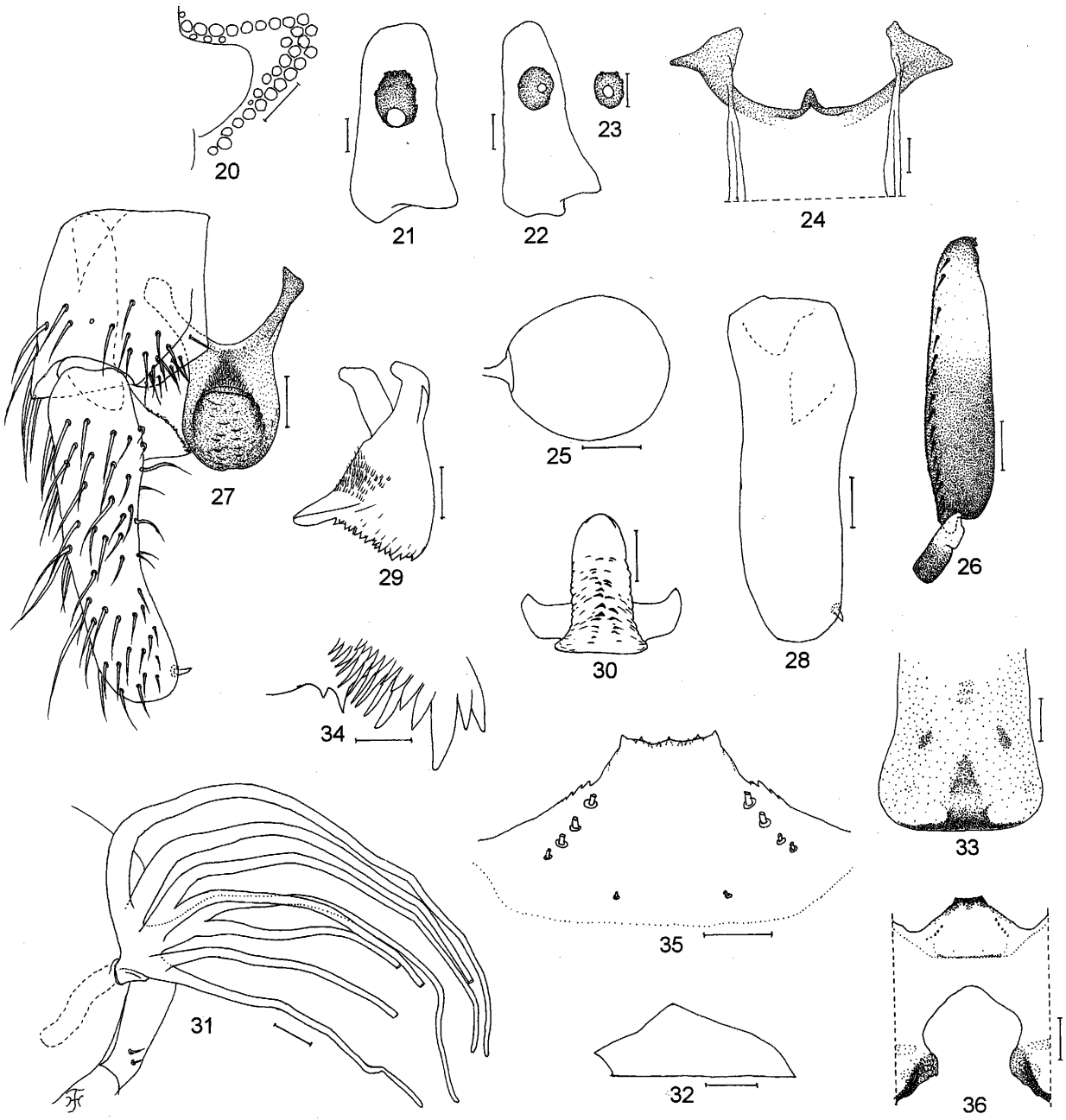
REMARKS. This species is assigned in the *multistriatum*-group of the subgenus *Simulium* (*Simulium*), redefined by Takaoka and Davies (1996), by the striated scutum, simple claws of female adult, horn-like basal protuberance of the male style (Fig. 11), and the 8-filamented pupal gill (Fig. 18).

S. chaliowae is easily distinguished from the other known species of this species-group by the shoe-shaped cocoon with an anterior collar of moderate height (Fig. 19) in place of the fenestrate cocoon. The female of this species appears to be similar to that of *S. hirtinervis* from Peninsular Malaysia (Takaoka and Davies, 1995) but differs by the much clearer coloring of the mid femur and tibia, the basal portion of radial vein with a few hairs near the apical end, and the smaller spermatheca. The male of *S. chaliowae* is similar to *S. fenestratum* from Sumatra and Thailand (Edwards, 1934; Takaoka and Saito, 1996), by having the hind basitarsus almost entirely darkened (Fig. 5) as well as the ventral plate hairy on ventral surface (Fig. 9), but differs from it by the horn-like basal protuberance of the style with many teeth along the anterior margin (Fig. 11).

***Simulium* (*Simulium*) *chainarongi* Kuvangkadilok and Takaoka sp. nov.**

Female. Body length ca. 2.6 mm. ***Head***. Narrower than thorax. Frons black, shiny, largely bare medially, with several dark hairs along each lateral margin and a few hairs above antennal base; frontal ratio 1.4:1.0:1.7; frons-head ratio 1.0:4.3. Fronto-ocular area (Fig. 20) moderately developed. Clypeus black, shiny, gray-pruinose, iridescent when illuminated, moderately covered with dark stout hairs except medial portion of upper 1/2 widely bare. Antenna composed of 2+9 segments, coloration differing between 2 ♀♀, i.e., in 1 ♀, brownish except scape, pedicel and 1st flagellar segment yellow, while in the other ♀, brownish except scape, pedicel and ventral surface of a few basal flagellar segments and dorsal surface of base of 1st flagellar segment yellow. Maxillary palp with 5 segments, brownish black, proportional lengths of 3rd, 4th and 5th segments 1.0:1.2:2.3; sensory vesicle (Fig. 21) ellipsoidal, ca. 0.35× as long as 3rd segment. Maxillary lacinia with 11 or 12 teeth on each side. Mandible with ca. 25 inner and 13 outer teeth. Cibarium (Fig. 24) with a blunt, medial projection on posterior margin, and without any tubercles. ***Thorax***. Nearly as in *S. chaliowae* ♀. ***Legs***. Foreleg: coxa and trochanter yellow; femur yellowish brown except basal 2/5 yellow; tibia white to whitish yellow except apical 1/5 black, with median large area white iridescent when illuminated; tarsus black, basitarsus dilated, ca. 4.9× as long as its greatest width. Midleg: coxa, trochanter and femur dark brown; tibia white with apical 1/5 dark brown; tarsus dark brown except basal 3/4 white. Hind leg: coxa dark brown; trochanter pale yellow; femur brown with apical cap dark brown and basal small area pale yellow; tibia white with apical 1/5 dark brown; tarsus dark brown except basal 2/3 of 1st segment and basal 1/2 of 2nd segment white; calcipala and pedisulcus well developed, as in *S. chaliowae* ♀. All tarsal claws simple. ***Wing***. Length 2.1 mm. Costa with spinules and hairs; subcosta haired except apical 1/4 or 1/5 bare; basal section of radial vein bare; hair tuft on stem vein brown. ***Abdomen***. Basal scale brown with fringe of pale long hair. Dorsal surface of 2nd segment pale with posterior 1/2 or less blackish, with a pair of dorsolateral silvery-iridescent spots broadly connected medially to each other; dorsal surface of remaining segments black, with short black hairs, with terga 6, 7 and 8 shiny; ventral surface of 7th segment with a pair of small, round sternal plates submedially. ***Genitalia***. Almost as in *S. chaliowae* ♀ except spermatheca nearly ovoid (Fig. 25).

Male. Body length 2.6 mm. ***Head***. As wide as thorax. Upper eye consisting of larger facets in 20 horizontal rows and in 20 vertical columns. Face and clypeus black, white-pruinose, silvery iridescent when illuminated, sparsely covered with long dark hairs. Antenna composed of 2+9 segments, brownish black except scape, pedicel and basal 2/5 of 1st flagellar segment yellow; 1st flagellar segment elongate, ca. 2.0× as long as 2nd one. Maxillary palp with 5 segments, brownish black, proportional lengths of 3rd, 4th and 5th segments 1.0:1.3:2.7; sensory vesicle (Figs. 22 and 23) small, nearly globular, with small opening. ***Thorax***. As in *S. chaliowae* ♂. ***Legs***. Foreleg: coxa whitish yellow; trochanter light brown on outer surface but yellow on inside surface; femur light brown to dark brown except basal 2/5 on inside surface dark yellow; tibia brownish



Figures 20-36 Morphological characters of *S.* (*S.*) *chainarongi* sp. nov. 20, ♀ fronto-ocular area; 21 and 22, 3rd segments of maxillary palp (21, ♀; 22, ♂); 23, sensory vesicle of a different ♂; 24, posterior part of ♀ cibarium; 25, spermatheca; 26, male hind basitarsus; 27, ♂ genitalia *in situ* (ventral view; left coxite, style and parameres omitted); 28, right style (ventrolateral view); 29 and 30, ventral plates (29, lateral view; 30, end view); 31, pupal gill filaments and interspiracular trunk (lateral view); 32, cocoon (lateral view); 33, larval cephalic apotome; 34, apical part of larval mandible; 35, larval hypostomium; 36, median portion of larval head capsule (ventral view) showing large postgenal cleft. Scale bars 1.0 mm for fig. 32; 0.1 mm for figs. 26, 31, 33 and 36; 0.05 mm for figs. 20, 25 and 35; 0.03 mm for figs. 27-30; 0.02 mm for figs. 21, 22, 23 and 34.

black with median large portion white on outer surface, which is iridescent when illuminated; tarsus brownish black, basitarsus moderately dilated, ca. 5.5× as long as its greatest width, and with a dorsal hair crest of moderate length. Midleg: brownish black except basal 2/3 of tibia and basal 1/2 of basitarsus white to whitish yellow; tibia largely white-iridescent on posterior surface when illuminated. Hind leg: coxa brownish black; trochanter yellow; femur brownish black with base narrowly yellow; tibia brownish black with base narrowly white; tarsus brownish black except basal 2/5 of basitarsus and basal 1/2 of 2nd segment dark yellow; basitarsus (Fig. 26) expanded, ca. 4.1× as long as its greatest width, and ca. 0.75× as wide as hind tibia which is subequal in greatest width to hind femur; calcipala and pedisulcus well developed. ***Wing***. Length 2.0 mm; other features as in ♀ except subcosta bare. ***Abdomen***. Basal scale brownish black with fringe of dark long hairs; dorsal surface of abdomen dark brown to brownish black, with dark short hairs, and terga 2, 5, 6 and 7 each with a pair of silvery iridescent large spots dorsolaterally; those on tergum 2 broadly connected in middle to each other. ***Genitalia*** (Figs. 27-30). Similar to those of *S. chaliowae* ♂ except slight differences in style and ventral plate. Style straight or only slightly curved inwards, widest subbasally, slightly narrowed toward midpoint, then nearly parallel-sided up to apical 1/6, ca. 3.1× as long as its greatest width, which is ca. 1.2× width at middle portion. Ventral plate in ventral view subquadrate but rounded posteriorly; posterior surface with ca. 20 teeth in 2 vertical irregular rows.

Pupa. Body length ca. 3.0 mm. ***Head*** and ***Thorax***. Integument as in *S. chaliowae* except coloring dark yellowish brown. Gill (Fig. 31) with 8 short, slender filaments in pairs, dorsal and ventral pairs short-stalked and 2 middle pairs medium-stalked, all lying almost in one vertical plane; an angle formed at base by dorsalmost and ventralmost filaments ca. 120 degrees; filaments somewhat decreasing in length (from ca. 1.2 mm to 0.8 mm) and thickness (at least basal portion) from dorsal pair to ventral one; 2 filaments of all pairs subequal in thickness to each other, except those of inner one of middle 2 pairs somewhat different; basal portion of 2 filaments of dorsal pair almost 2.0× as thick as that of 2 filaments of ventral pair, and slightly thicker than interspiracular trunk; all filaments light yellowish brown, tapered toward apex, with distinct annular ridges and furrows, and densely covered with minute tubercles. ***Abdomen***. As in *S. chaliowae* except tergum 7 bare. ***Cocoon*** (Fig. 32). Shoe-shaped, with anterior collar of variable heights, thickly woven, without open spaces in webs.

Mature larva. Body length 4.8-5.0 mm. Body color grayish black or greenish black. Cephalic apotome (Fig. 33) dark yellow to light yellowish brown except anterior 1/2 pale, narrowly darkened along posterior margin, and with positive head spots, though anteromedian spot faint, and posterolateral ones indistinct. Antenna composed of 3 segments and apical sensillum, longer than stem of labral fan; proportional lengths of 1st, 2nd and 3rd segments 1.0:1.2-1.4:0.9. Labral fan with ca. 42 main rays. Mandible (Fig. 34) with comb-teeth, decreasing in length from 1st to 3rd; mandibular serrations composed of 1 large and 1 small teeth, and without supernumerary serrations. Hypostomium (Fig. 35) with an anterior row of 9 teeth, all pointed apically; 2 outermost teeth of 6 intermediate teeth, as well as median and corner teeth, moderately developed; lateral serrations moderately developed; hypostomal bristles 4 or 5, slightly divergent posteriorly from lateral margin on each side. Postgenal cleft (Fig. 36) very large, rounded, constricted basally, and ca. 3.5× as long as postgenal bridge; elongate transverse spot on each side of postgenal cleft faintly positive. Thoracic cuticle bare. Abdominal cuticle with a dorsolateral pair of small protuberances each on segments 1-6, and with no distinct setae except last segment, moderately covered with short, colorless setae on each side of anal sclerite. Rectal gill compound, each of 3 lobes with 7-9 finger-like secondary lobules. Anal sclerite X-shaped, with anterior arms much shorter, ca. 0.6× as long as posterior ones. Accessory sclerite and ventral papillae absent. Posterior circlet with ca. 92 rows of up to 16 hooklets per row.

TYPE SPECIMENS. Holotype ♀, reared from pupa, collected from Klang Lum Duan, Yod Dome Wild Life Sanctuary, Ubonratchathani Province, northeastern Thailand, 16. IX. 1998, by Chaliow Kuvangkadilok and Chainarong Boonkemtong. Paratype 2 ♀♀, reared from pupa, 1 ♂, reared from pupa, and 3 mature larvae, same data as holotype.

ECOLOGICAL NOTES. This species was collected from trailing grasses, roots and fallen leaves in a stream 2-10 m wide with water temperature of 26.5°C, at Klang Lum Duan (140 m in altitude). This species was also found in Huai Luang waterfall (300 m in altitude), where the stream was fast flowing, shaded, and 5 m wide, and the water temperature was 24°C. This species

was collected together with *S. nakhonense, S. parahiyangum, S. siamense* and *S. asakoae.*

ETYMOLOGY. The species *chainarongi* is named after Mr. Chainarong Boonkemtong.

REMARKS. This new species resembles *S. chaliowae* by having the shoe-shaped cocoon with an anterior collar, as well as the similar genitalia of both sexes of adults, but is distinguished from the latter species in the female by the mid and hind femora almost entirely darkened, in the male by the hind basitarsus dark yellow on the basal 2/5 and brownish black on the apical 3/5 (Fig. 26), the style not narrowed medially (Fig. 28) and the ventral plate with more numerous teeth on the posterior surface (Fig. 30), and in the pupa by the dorsalmost filament twice as thick in the basal portion as the lowermost one, and subequal to, or slightly thicker than, the interspiracular trunk (Fig. 31). The larva of *S. chainarongi* is similar to that of *S. hirtinervis* Edwards, from Peninsular Malaysia (Takaoka and Davies, 1995) by having the dorsal protuberances on the abdomen. However in the latter species these protuberances are present not only on the abdominal segments 1-6 but also on the segments 7 and 8.

***Simulium* (*Simulium*) *triglobus* Takaoka and Kuvangkadilok sp. nov.**

Female. Body length 2.6 mm. ***Head***. Narrower than thorax. Frons brownish black, shiny, with several dark stout hairs along lateral margins; frontal ratio 1.6:1.0:1.4; frons-head ratio 1.0:3.7. Fronto-ocular area well developed, similar to that of *S. chaliowae* ♀. Clypeus brownish black, shiny, gray-pruinose, iridescent when illuminated, moderately covered with dark stout hairs. Antenna composed of 2+9 segments, yellow on scape, pedicel and a few basal flagellar segments, and brownish on rest flagellar segments, its border not well defined. Maxillary palp with 5 segments, light brown, proportional lengths of 3rd, 4th and 5th segments 1.0:1.2:2.4; 3rd segment (Fig. 37) of normal size, with sensory vesicle ellipsoidal, ca. 0.36× as long as 3rd segment. Maxillary lacinia with 15 inner teeth and 13 or 14 outer teeth. Mandible with ca. 24 inner teeth and ca. 10 outer teeth. Cibarium as in *S. chainarongi* ♀. ***Thorax***. Similar to that of *S. chainarongi* ♀ except scutellum dark yellow, postscutellum dark brown, and recumbent pubescence on scutum and scutellum yellow. ***Legs***. Foreleg: coxa, trochanter and femur entirely yellow; tibia white except apical 1/5 brownish black, with large area of white sheen on outer surface; tarsus brownish black; basitarsus somewhat dilated, ca. 5.3× as long as its greatest width. Midleg: coxa blackish brown; trochanter and femur entirely yellow; tibia white to yellowish white except apical tip dark yellow, with large area of white sheen on posterior surface; tarsus blackish brown except basal 1/2 of basitarsus whitish yellow. Hind leg: coxa blackish brown; trochanter and femur entirely yellow, with apex of femur slightly darkened; tibia white to yellowish white with apical 1/5 brownish black, with large area of white sheen on posterior surface; tarsus blackish brown except basal 1/2 or a little more of basitarsus and basal 1/2 of 2nd segment yellowish white; basitarsus parallel-sided; calcipala and pedisulcus moderately developed. All tarsal claws simple. ***Wing***. Length ca. 2.5 mm. Costa with spinules and hairs; subcosta haired except apical 1/5 or 1/3 bare; basal section of radial vein bare; hair tuft on stem vein brown. ***Abdomen***. Basal scale dark yellow to light brown, with fringe of pale long hairs, of which basal portions are dark. Dorsal surface of 2nd segment pale yellowish white with narrow area just before posterior margin brownish black; dorsal surface of 3rd to 6th segments brownish black, and that of remaining posterior segmnts light yellowish brown mottled with many blackish spots, with short dark hairs; terga 6-8 shiny. Ventral surface of 7th segment with a pair of small submedial sternal plates. ***Genitalia*** (Figs. 38-42). Sternite 8 moderately sclerotized, narrowly bare in middle, covered with 32-36 stout hairs on each lateral side. Anterior gonapophyses triangular, moderately separated from each other medially, membraneous, covered with numerous microsetae and 3-5 stout hairs per each; narrow bare area along inside and posterior margins very thin and transparent (then often overlooked). Genital fork of inverted-Y form, with narrow, well sclerotized stem, which is at base widened and angulated against arms, as seen laterally (Fig. 42); arms broad basally, each with distinct wide plate-like projection directed anteriorly. Paraproct much protruding ventrally, 1.8-2.0× as wide as long, with numerous stout hairs on lateral and ventral surfaces. Cercus short, ca. 2.0× as wide as long, with numerous stout hairs, with rounded or medially slightly concave posterior margin, when viewed laterally (Figs. 39 and 40). Spermathecae 3 in number, all well sclerotized and with internal setae, but differing in size and shape; 1 principal spermatheca, which has longer spermathecal duct, is large and pear-shaped, and 2 accessory ones, both of which bear somewhat shorter spermathecal duct, are equally small, oblong and curved.

Male. Unknown.

Pupa. Body length ca. 2.8 mm. ***Head*** and ***Thorax***. Integument dark yellow to light yellowish brown, densely and elaborately covered with round tubercles (cone-shaped tubercles on posterodorsal surface of thorax); head with 1 facial pair of bifid medium trichomes, and 2 frontal pairs of bifid and trifid medium trichomes widely spaced; thorax on each side with 3 medium trichomes each with 4-5 branches mediodorsally, 1 long trichome with 5 branches and 1 medium trichome with 4 branches anterolaterally, 1 long trichome with 2-4 branches posterolaterally, and 1 medium trichome with 3 or 4 branches and 2 bifid long trichomes ventrolaterally, all long trichomes rather stout. Gill (Fig. 43) with 8 short, slender filaments in pairs (all short-stalked), widely diverging vertically but not so horizontally, with an angle formed at base by dorsalmost and ventralmost filaments nearly 140 degrees; filaments somewhat decreasing in length (from ca. 1.0 mm to 0.6 mm) from dorsal pair to ventral one, and also slightly decreasing in thickness from dorsal pair to ventral one, dorsalmost filament is ca. 1.3× as thick as lowermost one, and ca. 0.7× as thick as interspiracular trunk; all filaments unpigmented or light yellow, tapered toward apex, with distinct annular ridges and furrows forming elaborate reticulate surface patterns, and densely covered with minute tubercles. ***Abdomen***. Tergum 1 light brown, bare, with 1 bifid medium seta on each side; tergum 2 pale, with 1 bifid medium seta and 5 short spines (of which 4 are much stouter) on each side; terga 3 and 4 each with 4 hooked spines and 1 simple short spinous seta on each side; tergum 8 with distinct spine-combs in transverse row on each side; terga 5, 6, 7 and 9 bare; terminal hooks absent. Sternum 5 with a pair of simple inner and bifid outer hooks submedially on each side; sterna 6 and 7 each with a pair of simple inner and outer hooks widely spaced on each side. ***Cocoon*** (Fig. 44). Shoe-shaped with anterior collar of moderate height, roughly woven anteriorly, then leaving some large open spaces in webs of anterior collar, and also many small open spaces near anterior margin; no ventrolateral extension.

Mature larva. Body length 5.4-6.0 mm. Body color greenish brown or grayish black. Cephalic apotome pale yellowish white (though anterior 1/2 and along right lateral margin somewhat darkened in 1 larva), and along posterior margin narrowly light brown; head spots indistinct (though in 1 larva posteromedian longitudinal spot and mediolateral round spots are faintly discernible). Antenna composed of 3 segments and apical sensillum, longer than stem of labral fan; proportional lengths of 1st, 2nd and 3rd segments 1.0 : 1.5 : 0.7. Labral fan with 56-60 main rays. Mandible (Fig. 45) with comb-teeth, decreasing in length from 1st to 3rd; mandibular serrations composed of 1 large and 1 medium teeth, and without supernumerary serrations; most teeth blunt, and rounded apically. Hypostomium (Fig. 46) with an anterior row of 9 teeth, all rounded apically; 2 outermost teeth of intermediate teeth, as well as median and corner teeth, moderately developed; lateral serrations moderately developed; hypostomal bristles 5-7, somewhat divergent posteriorly from lateral margin on each side. Postgenal cleft (Fig. 47) large, rounded anteriorly, not constricted basally, ca. 2.0× as long as postgenal bridge; elongate transverse spot on each side of postgenal cleft negative. Thoracic cuticle bare. Abdominal cuticle without dorsolateral protuberances and distinct setae except last segment moderately covered with short, colorless setae on each side of anal sclerite. Rectal gill compound, each of 3 lobes with 10-13 finger-like secondary lobules. Anal sclerite X-shaped, with anterior arms much shorter, ca. 0.6× as long as posterior ones. Accessory sclerite and ventral papillae absent. Posterior circlet with 112-134 rows of up to 22 hooklets per row.

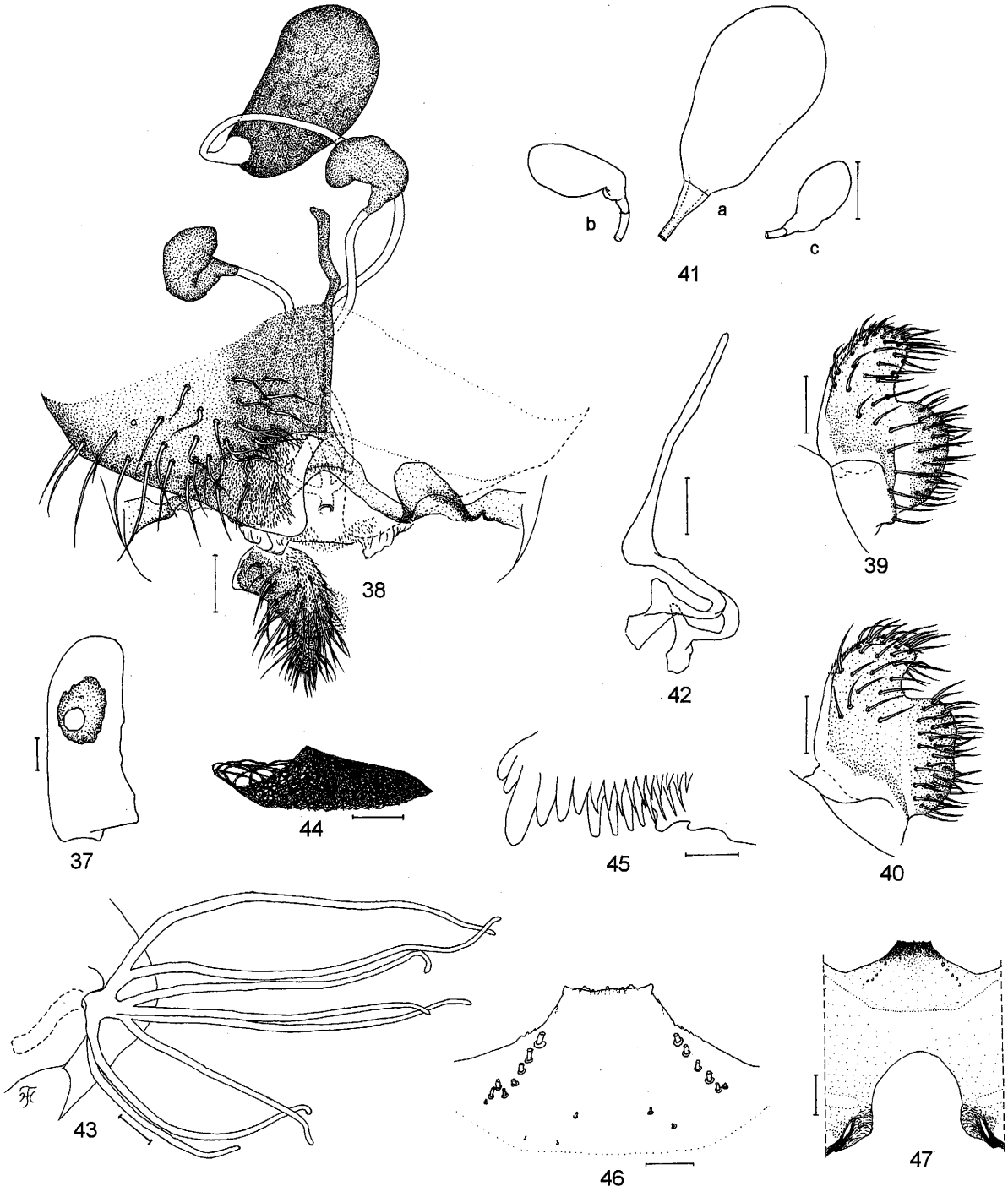
TYPE SPECIMENS. Holotype ♀, reared from pupa, collected from Tontong waterfall, Doi Phuka National Park, Nan Province, northern Thailand, 9. XII. 1998, by Chaliow Kuvangkadilok and Chainarong Boonkemtong. Paratype 1 ♀, reared from pupa, 1 pupa, and 2 mature larvae, same data as holotype.

ECOLOGICAL NOTES. This species was collected from fallen leaves in a small stream 0.5-1.0 m wide at Tontong waterwfall (altitude 500 m), where the water temperature was 19°C. It was found with *S. rufibasis*.

ETYMOLOGY. The species name *triglobus* refers to the three spermathecae.

REMARKS. This new species is also placed in the *multistriatum*-group within the subgenus *Simulium* (*Simulium*) by the striated scutum and simple claws of female adult, and the 8-filamented pupal gill.

The pupa of *S. triglobus* is easily separated from the other known species of the same species-group, by the cocoon of corbicular shape (Fig. 44). The pupa of this species also differs from those of *S. chaliowae* and *S. chainarongi*, described above, by the more branched



Figures 37-47 Morphological characters of *S.* (*S.*) *triglobus* sp. nov. 37, 3rd segment of ♀ maxillary palp; 38, ♀ genitalia *in situ* (ventral view) showing 1 principal and 2 accessory spermathecae; 39 and 40, paraprocts and cerci (39, holotype; 40, paratype); 41, 3 spermathecae (a, principal; b and c, accessory) of paratype ♀; 42, genital fork (lateral view); 43, pupal gill filaments and interspiracular trunk (lateral view); 44, cocoon (lateral view); 45, apical tip of larval mandible; 46, larval hypostomium; 47, median portion of larval head capsule (ventral view) showing postgenal cleft. Scale bars 1.0 mm for fig. 44; 0.1 mm for figs. 43 and 47; 0.05 mm for figs. 38-42 and 46; 0.02 mm for figs. 37 and 45.

thoracic trichomes, as well as the corbicular cocoon, and from *S. chainarongi* by the slenderer gill filaments (Fig. 43).

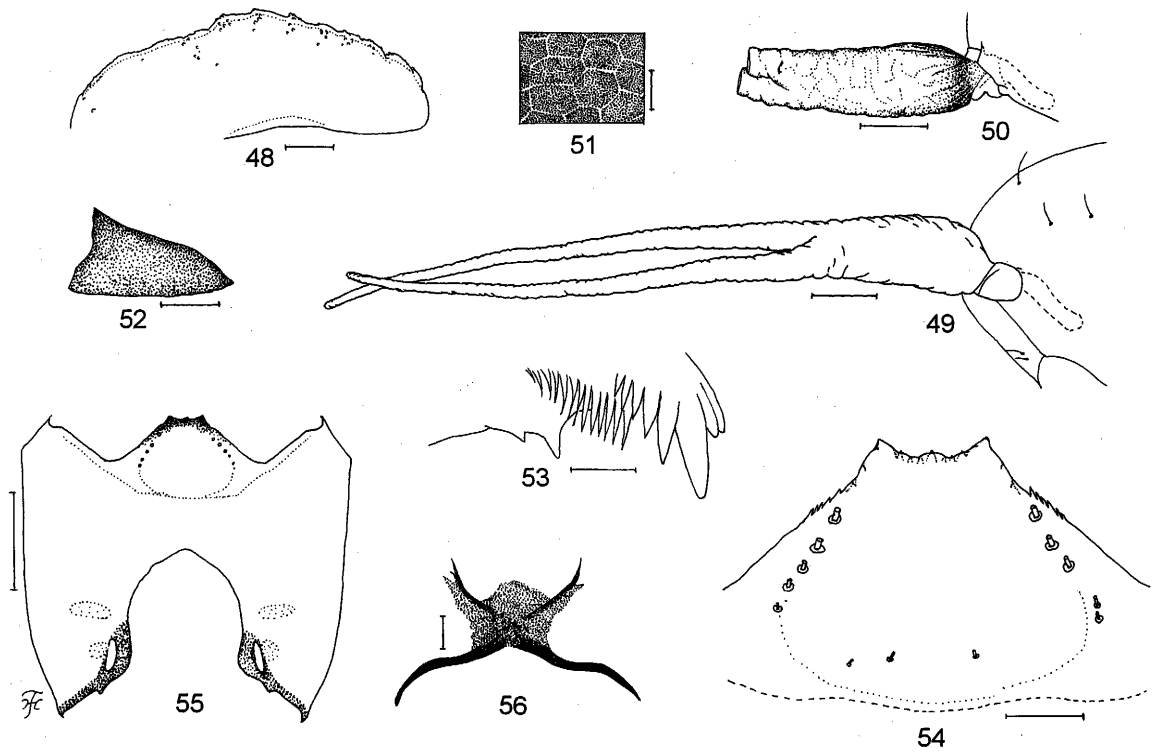
The female of this species differs from the former two related species by the clearer leg coloring, and the genital fork with a wide plate-like projection directed forwards on each arm (Fig. 38). However, the most striking character found in this new species is the presence of two more spermathecae in addition to the single, typical one (Figs. 38 and 41). All other black-fly species have a single spermatheca (Crosskey, 1990), though they retain two additional spermathecal ducts arising from the main spermathecal duct near its base. Both additional ducts are, however, much shorter than the main one, as shown in Fig. 6. Three spermathecae are considered to be the evolutionarily basic complement for the Diptera (Downes, 1968). In other nematocerous insects, such as mosquitoes and biting midges, the number of spermathecae has been known to vary from one to three across genera or by species (Lane and Crosskey, 1993). Therefore, it was not unexpected to encounter a species carrying two or three spermathecae even in the small family Simuliidae. Our finding is the first to record a species with three spermathecae in the Simuliidae. Bernard (1974) reported two spermathecae of almost the same size in a freak specimen of *S. erythrocephalum*. Hunter and Adler (pers. commun.) also found aberrant females with two spermathecae of different size from colony-reared *S. vittatum*.

***Simulium baimaii* Kuvangkadilok and Takaoka sp. nov.**

Female and Male. Unknown.

Pupa. Body length (excluding gill filaments) 3.1 mm. ***Head.*** Integument light yellowish brown, moderately covered with round tubercles; antennal sheath (Fig. 48) with 8 small ridges on outer surface, each of which is sparsely covered with round tubercles; face on each side with 1 simple long slender trichome; frons on each side with 2 simple long slender trichomes widely separated from each other. ***Thorax.*** Integument light yellowish brown, sparsely covered with round tubercles except anterior portion moderately covered with similar tubercles; thoracic trichomes all simple, long and slender—2 dorsomedially, 2 anterolaterally, 1 posterolaterally, and 3 ventrolaterally. Gill (Figs. 49 and 50) with 2 filaments arising from inflated trunk, ca. 2 mm long from its base to apex of filaments, and with somewhat swollen bulb at base; 2 filaments subequal in size to each other, gradually tapered toward apex; surface of filaments and basal inflated portion light yellowish brown and thick (except bulb at base pale and thin), and irregularly wrinkled with many transverse or oblique furrows, and densely covered with minute tubercles, elaborately forming numerous small reticulate surface patterns near base, as shown in Fig. 51. ***Abdomen.*** Tergum 1 sparsely tuberculate, with 1 simple long slender seta on each side; tergum 2 with 1 simple long slender seta and 5 spinous setae, on each side; terga 3 and 4 each with 4 distinct hooked spines directed forward on each side; terga 5 and 6 without spine-combs, but terga 7–9 each with distinct spine-combs directed backward in transverse row, together with numerous comb-like groups of minute spines, on each side; terminal hooks absent. Sternum 5 with 2 bifid hooks submedially, on each side; sterna 6 and 7 each with 1 bifid hook submedially and 1 bifid or simple hook laterally, on each side; sterna 4–8 each with numerous comb-like groups of minute spines; sternum 9 bare. ***Cocoon*** (Fig. 52). Simple, wall-pocket-shaped, light brown, intactly woven leaving no open spaces in the wall, and with no ventrolateral extension; individual threads invisible; 2.8 mm long × 1.0 mm wide.

Mature larva. Body length 6.0–6.5 mm. Body color yellowish. Cephalic apotome yellow to yellowish brown with median small area brown between posterolateral spots just before posterior margin; head spots brown except posterolateral spots pale showing negative pattern. Antenna with 3 segments and apical sensillum, longer than stem of labral fan; proportional lengths of 1st, 2nd and 3rd segments 1.0:1.2:0.7. Labral fan with ca. 60 main rays. Mandible (Fig. 53) with comb-teeth decreasing in size from 1st to 3rd; mandibular serrations composed of 1 large and 1 small teeth, without supernumerary serrations. Hypostomium (Fig. 54) with an anterior row of 9 teeth; each corner tooth moderately developed; median tooth and intermediate teeth bent to some extent dorsally, then invisible from the ventral side; hypostomal bristles 5 or 6 in number, slightly divergent posteriorly from lateral margin on each side. Postgenal cleft (Fig. 54) well developed, round apically, longer than wide, ca. 2.5× as long as postgenal bridge. Thoracic cuticle bare. Abdomen gradually enlarged posteriorly from 1st to 8th segment and abruptly tapered toward posterior tip; abdominal cuticle almost bare except last segment moderately covered with short, colorless setae dorsally and laterally. Rectal gill compound, each of 3 lobes with 12–14 finger-like secondary lobules. Anal sclerite (Fig. 56) X-shaped, with its central portion and area between anterior arms broadly



Figures 48-56 Morphological characters of *S. baimaii* sp. nov. 48, pupal antennal sheath (lateral view); 49, pupal gill and interspiracular trunk (lateral view); 50, basal 1/3 of pupal gill (dorsal view); 51, reticulate pattern on the surface of inflated portion of pupal gill; 52, cocoon (lateral view); 53, apical tip of larval mandible; 54, larval hypostomium; 55, larval head capsule (ventral view) showing postgenal cleft; 56, anal sclerite. Scale bars 1.0 mm for fig. 52; 0.2 mm for figs. 49, 50 and 55; 0.05 mm for figs. 48, 54 and 56; 0.02 mm for fig. 53; 0.01 mm for fig. 51.

sclerotized; anterior arms broad, and ca. 0.7× as long as posterior ones; accessory sclerite and ventral papillae absent. Posterior circlet with 90-102 rows of up to 16 hooklets per row.

TYPE SPECIMENS. Holotype pupa, collected from Phra Aung waterfall, Phu Kradung National Park, Loei Province, northeastern Thailand, 25. X. 1995 and 23. V. 1996, by Narumon Sangpradub and Chutima Hanjavanit. Paratype 2 mature larvae, same data as holotype.

ECOLOGICAL NOTES. This species was found on stones in a fast flowing stream 3 m wide at Phra Aung waterfall (altitude 1,124 m). No other species was found with this new species.

ETYMOLOGY. The species *baimaii* is named after Prof. Visut Baimai.

REMARKS. *S. baimaii* sp. nov. is easily assigned to the genus *Simulium* s.l., defined by Crosskey (1969), by the normal onchotaxy of the pupal abdomen and the shape of the cephalic apotome, hypostomal teeth and mandibular serrations of the larva. However, its subgeneric assignment is not made due to lack of adult specimens.

The pupa of *S. baimaii* is characterized by the gill having two filaments on each side (Fig. 49), a character not found in Simuliidae of the Oriental Region, though *S. (Nevermannia) taulingense* from Taiwan has the pupal gill composed of two bulbs (Takaoka, 1979). Similar reduced number of gill filaments (i.e., two), though different in shape, has been reported in one species of the subgenus *Simulium (Byssodon)* from Africa (Crosskey, 1969) and in several species of the subgenus *Simulium (Hearlea)*, and in one species of the genus *Mayacnephia,* from Central America (Dalmat, 1955).

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A SURVEY ON HELMINTHIC INFECTIONS IN TWO RURAL COMMUNITIES IN NEPAL

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Abstract: This study was carried out to elucidate the prevalence of intestinal helminthic infections in rural communities in Nepal. Of 231 inhabitants randomly sampled in Kotyang and Judigaun, 140 (60.6%) were found to be infected with some soil-transmitted helminths. The highest prevalence was observed in hookworm infection (52.8%), followed by *Trichuris trichiura* (18.6%) and *Ascaris lumbricoides* (11.3%) infections. Some inhabitants harboured *Vampirolepis nana* and liver fluke. The female group aged 60 years old and more showed significantly higher *T. trichiura* infection rate than the male group with the same age ($p<0.05$), while no relationship was detected between proportion of *T. trichiura* infection and age based on logistic regression test ($p=0.07$). Serum IgE levels of Nepalese were shown to be far higher than common Japanese levels, suggesting the repeated infections with these helminths.

Key words: Nepal, intestinal parasite, IgE, epidemiology

INTRODUCTION

Soil-transmitted helminths or geo-helminths are of great importance in the health sector in developing countries with poor socio-environmental conditions. It is estimated that some 3.5 billion people are infected with these helminths (Bundy *et al.*, 1996; Chan *et al.*, 1994; Chan, 1997) in the world. Mixed infections with several different parasites (e.g., hookworms and *A. lumbricoides*) are commonly seen. Although the mortality rate by these infections is rather low, these infections are recognized as a serious public health problem because of the high prevalence (Bundy *et al.*, 1996; Chan *et al.*, 1994; Chan, 1997). *A. lumbricoides* may contribute to protein-energy malnutrition, retarded physical activity and poor growth (Adams *et al.*, 1994). Hookworm or *T. trichiura* contributes to iron deficiency anemia (Stoltzfus *et al.*, 1996; Roche and Layrisse, 1966).

Since Nepal currently has a population of over 23 million and most of the people live in rural areas, it is important to elucidate the prevalence of soil-transmitted helminthic infections in rural communities for the future control. Thus the present study was undertaken to estimate the prevalence in two rural communities.

MATERIALS AND METHODS

This study was performed in two rural communities (Kotyang and Judigaun) located in a hill area of Kabhrepalanchok District, 30 km away from Kathmandu, the capital of Nepal, from February to March, 1998. Kotyang is in Anaikot Village Development Committee, the settlement of which extends from 1,100 to 1,300 m in altitude where malaria is not endemic. The other locality is Judigaun in Mahadevstan Village Development Committee located in a hot valley where malaria has been endemic. The altitude is ca. 900 m. The inhabitants of Kotyang are mainly composed of hill peoples such as the Tamangs and the Parbate Hindus, whereas those of Judigaun are the Rai Danuwars. The inhabitants of Kotyang usually eat rice, corn, wheat flour, potato, green and other vegetables, milk, chan, which is

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Table 1 Age and sex distribution of inhabitants examined

Age (in years)	Male	Female	Total
10-19	10 (8.9%)	13 (10.9%)	23 (10.0%)
20-29	33 (29.5%)	38 (31.9%)	71 (30.7%)
30-39	26 (23.2%)	19 (16.0%)	45 (19.5%)
40-49	8 (7.1%)	20 (16.8%)	28 (12.1%)
50-59	9 (8.0%)	10 (8.4%)	19 (8.2%)
60<	26 (23.2%)	19 (16.0%)	45 (19.5%)
Total	112 (100%) (48.5%)	119 (100%) (51.5%)	231 (100%) (100%)

a local alcoholic drink, other beverages, and a small amount of meat, egg, sugar and oil (Ito *et al.*, 1993), whereas those of Judigaun sometimes eat freshwater fish in addition to above-mentioned foods. Most houses have a lavatory. Fecal and blood samples were collected in these communities. Feces of 231 inhabitants (10 years old and more) in Kotyang and Judigaun (Table 1) were examined for intestinal helminthic infections by MGL method after fixation in 10% formalin. Sera collected and preserved under −20°C were transferred to Japan, and the total serum IgG and IgE were measured by ELISA for 175 samples at CRC Co. (Fukuoka, Japan). Before survey, we obtained consent of community leaders and villagers. The association between the infection rate and age was tested with the logistic regression. Likelihood ratio test based on the logistic model was used to test the sexual difference in the relationship between the infection rate and age. These statistical analyses were conducted with Stata Release 6.0 (Stata Corp). The χ^2 test was performed where it is applicable.

RESULTS

Of 231 inhabitants, 140 (60.6%) were infected with soil-transmitted helminths. The pattern of infection in two communities was quite similar (Fig. 1). The highest prevalence was observed in hookworm infection, followed by *T. trichiura* and then *A. lumbricoides* infections. Hundred twenty-two of inhabitants (52.8%) were with hookworm, 43 (18.6%) with *T. trichiura* and 26 (11.3%) with *A. lumbricoides* (Fig. 1). Mixed infections with hookworm plus *Trichuris* were the commonest (Fig. 2). Male and female inhabitants showed similar prevalence in any of these three helminth infections (data not shown). The infection rate of *T. trichiura* was gradually decreased with age in males. In females, on the other hand, the *Trichuris* egg-positive rate was found higher after 40 years old and more and it increased with age. Although no relationship was detected between age and prevalence based on logistic regression test ($p=0.07$), female group aged 60 years old and more showed higher infection rate than the same age group of males (χ^2 test: $p<0.05$, Fig. 3). In Judigaun, some inhabitants were found to be infected with *V. nana* and liver fluke (Fig. 1).

We examined the total serum IgG and IgE levels of the inhabitants in these two communities. The majority of total serum IgG levels of the inhabitants were high in both sexes, 2,273±876 mg/d*l* in male and 2,024±499 mg/d*l* in female in average. With regard to total serum IgE levels, all the inhabitants surpassed Japanese normal range (<165 IU/m*l*) both in male and female, irrespective of excretion of parasite eggs (Fig. 4). Total IgE levels in inhabitants infected with either hookworm

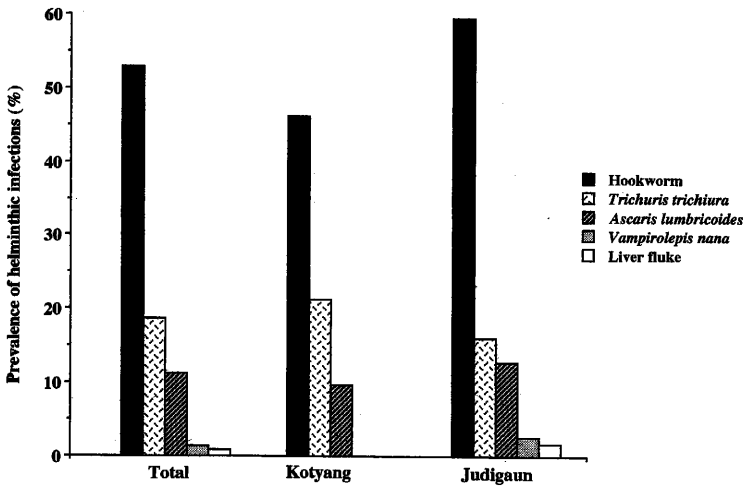

Figure 1 Prevalence of helminthic infections at Kotyang and Judigaun in Nepal.

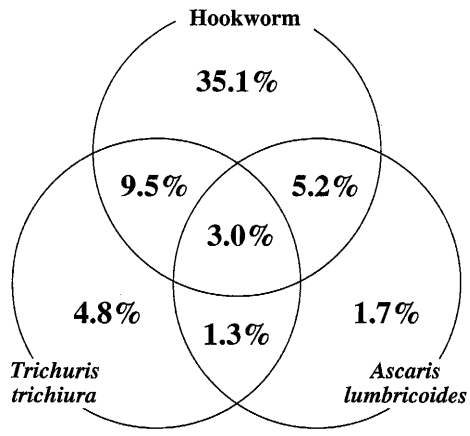

Figure 2 The status of multiple infections with helminths at Kotyang and Judigaun in Nepal.

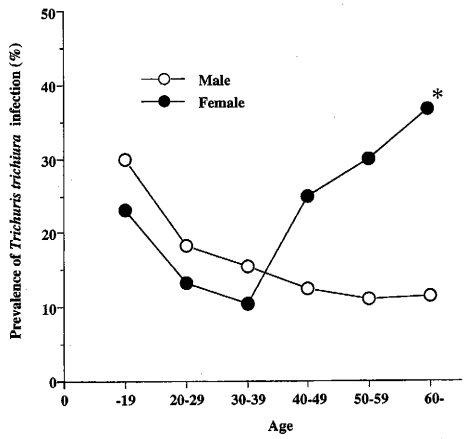

Figure 3 Prevalence of *T. trichiura* infection by age at Kotyang and Judigaun in Nepal. *p<0.05 compared with male group.

or *T. trichiura* ranged from 200 to 3,000 IU/m*l* (an upper limit in this assay without dilution), while all infected with *A. lumbricoides* showed higher than 1,500 IU/m*l* (Fig. 5).

DISCUSSION

It is reported that the prevalence of soil-transmitted helminthic infection at Kathmandu, the capital city of Nepal, ranged from 18.0 to 36.6%, and that the annual prevalence was decreasing year by year in both adults and children, irrespective of sex (Rai *et al.*, 1994). Our study was carried out in two rural communities of a hill area, Kotyang and Judigaun, which were located at 30 km north-east of Kathmandu. It was elucidated that the prevalence and the species of parasite prevailed in these rural villages were different from Kathmandu. In both Kotyang and Judigaun, the prevalence of soil-transmitted helminthic infections was higher than 60%, three times as high as that (less than 20%) in Katmandu in 1992 (Rai *et al.*, 1994).

The most common helminth was hookworm (52.8%), followed by *T. trichiura* (18.6%) and *A. lumbricoides* (11.3%) in these two communities. It is worthy of notice that *Ascaris* infection rate in our survey was rather low (11.3%) in both Kotyang and Judigaun compared with other place in Nepal (Rai and Gurung, 1986; Rai *et al.*, 1994; Rai *et al.*, 1997; Gianotti, 1990; Navitsky *et al.*, 1998). The pattern of infection was also different from those of other areas in Nepal including Kathmandu, where the most common helminth was *A. lumbricoides,* followed by hookworm and *T. trichiura* (Rai and Gurung, 1986; Rai *et al.*, 1994; Rai *et al.*, 1997; Gianotti, 1990). The study conducted in rural areas of Sarlahi District, low plains adjacent to India, showed that the prevalence rates of helminth infection were 78.8%, 56.2%, and 7.9% in hookworm, *A. lumbricoides,* and *T. trichiura,* respectively (Navitsky *et al.*, 1998).

It is a well known fact that there are remarkable differences in the detection of hookworm infection rate depending on the examination methods employed. The direct fecal smear method reveals rather poor result in eggs detection in comparison with the floatation, precipitation and cultivation methods (Kamegai, 1972). Therefore it should be noted that the results by the

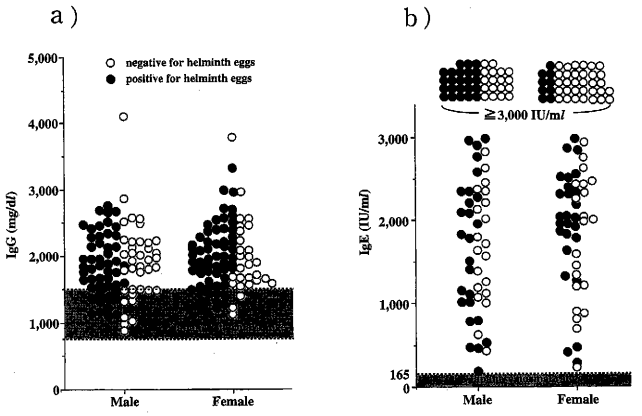

Figure 4 Total serum IgG and IgE levels in males and females at Kotyang and Judigaun in Nepal. Total serum IgG (a) and IgE (b) levels in males and females who were negative (open symbol) or positive (closed symbol) for helminths eggs.

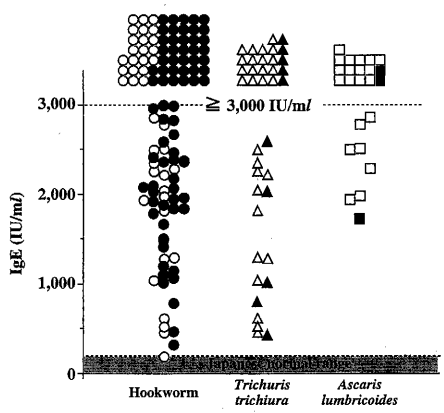

Figure 5 Total serum IgE levels of inhabitants infected with hookworms (circle), *T. trichiura* (triangle) and *A. lumbricoides* (square). Total serum IgE levels of inhabitants infected with a single helminth (hookworm, *T. trichiura* or *A. lumbricoides*) were shown as closed symbols and those infected with 2 or 3 helminths were shown as open symbols.

direct smear method (Rai and Gurung, 1986; Rai *et al.*, 1994; Rai *et al.*, 1997) had resulted in considerably lower rate than true infection for hookworm. Concerning the hookworm species prevalent in Nepal, in Sarlahi District, all of the cultured hookworm larvae were identified as *Ancylostoma duodenale* (Gianotti, 1990). In Kathmandu, 67.0% of hookworm were *A. duodenale,* while 33.0% were *Necator americanus* (Rai *et al.*, 1997). In our survey, we did not adopt the cultivation method and so that could not differentiate the species. The infection rate of *T. trichiura* in Nepal was rather low ranging between 5 and 20% (Rai and Gurung, 1986; Rai *et al.*, 1994; Rai *et al.*, 1997; Gianotti, 1990; Navitsky *et al.*, 1998). In Judigaun, some inhabitants were found to be infected with *V. nana* and liver fluke (Fig. 1). As *T. trichiura* is a typical "backyard" pathogen, a high prevalence with this parasite in females will be attributed to house works and consequent contaminations, too.

With regard to total serum IgE level, all inhabitants surpassed Japanese normal range (<165 IU/m*l*) both in male and female, irrespective of excretion of parasite eggs (Fig. 4). This result indicates the occurrence of repeated infections with helminths. The discrepancy between serum IgE level and excretion of parasite eggs might be accounted for by the fact that the time and place of sensitization of immune response against helminth antigens are different from those of egg-production from adult helminths. In this study, it was shown that total IgE levels in inhabitants infected with hookworm or *T. trichiura* ranged from 200 to higher than 2,000 IU/m*l* (upper limit of measurement, 3,000 IU/m*l*), while all infected with *A. lumbricoides* were with IgE higher than 1,500 IU/m*l* (Fig. 5). Thus this fact may support previous estimation that the *Ascaris* infection would strongly induce IgE antibody production (Kojima *et al.*, 1972).

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Jpn. J. Trop. Med. Hyg., Vol. 27, No. 4, 1999, pp. 517-519

Short communication

PREVALENCE OF INTESTINAL PARASITE INFECTION IN TOUL ROKA VILLAGE, PHNOM PENH MUNICIPALITY, CAMBODIA

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Intestinal parasitic infection is a serious public health problem among the inhabitants in developing countries located in tropical areas. Control activities of parasitic diseases are not only an important health improvement problem in a given area but also an entry point for effective public health care activities because of its serious prevalence among inhabitants and its nature as an environment-derived disease (Fereydoun, 1984). Also, intestinal helminthiasis can be easily diagnosed with fecal samples and are treatable by oral administration of anthelmintics; it can be performed intensively as an ordinary public health care activity. Additionally, intestinal parasitic infection occurs closely relating to the environmental sanitary condition; parasite control program may relate directly to the education for improvement of sanitary condition. Exact survey on the prevalence of intestinal parasite infection may also be important to know health status of the inhabitants in a given area. Due to the civil war for a long period in Cambodia, the information on the recent prevalence of intestinal parasites is extremely few. In the country, Cambodia-Okinawa Friendship Association (COFA), founded in 1992 as a non-government organization (NGO) in Okinawa, has established a clinic in a village, Toul Roka Village, to contribute to health care of the villagers and started a parasite control program since 1998. The present study was undertaken to obtain basic information on the recent prevalence of intestinal parasitic infection among the inhabitants in the village.

Although the village is located in suburban area of Phnom Penh Municipality, the general socio-economic and sanitary conditions were consistently poor in the village. A total of 457 villagers live in 61 households in the village. The survey was conducted by stool examination in December, 1998. For stool examination, the authors visited all households to explain the purpose of the survey and delivered stool containers to all families. After delivering the containers, stool samples were collected and examined both by Kato-Katz thick smear method (Kato and Miura, 1954) and formalin-ether concentration method (Ritchie, 1948). Their life habits were also surveyed by the interview to the representative adults of each family.

In the present preliminary study, stool samples from 113 villagers, accounting for 24.7% of the population subjected, were collected randomly for examination. Table 1 represents the results of the stool examinations. A total of 7 helminth and 9 protozoan species were diagnosed in 80.5% of the villagers examined. Sixty-seven samples (59.3%) were found harboring one or more helminth infections. Among the helminth species, *Ascaris lumbricoides* was the commonest parasite, showing 48.7% of prevalence rate. Hookworm infection was also demonstrated in 15.9% of the villagers. On the other hand, *Trichuris trichiura* infection was rare among the villagers, although their infection mode is almost the same to that of *A. lumbricoides*. Interestingly, the infection rate with *Opisthorchis viverrini*, liver fluke, were only 3.5% (4 cases), in spite of frequent eating of raw fishes by the villagers. When the prevalence rates were compared between age groups divided by 15 years old, the infection rate of *A. lumbricoides* was higher in the younger age population, but it was reversed by the aged group over 15 years old in the case of hookworm infection. As to protozoan parasites, 9 species of protozoan parasite were detected in 61 (54.0%) villagers. The commonest species was *Blastcystis hominis*, showing positive rates of 37-43%. *Entamoeba histolytica/E.*

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Table 1 Prevalence of intestinal helminth infection in Toul Roka village, Cambodia

	No. of positive (%)		
	Under 15 years old (n=51)	15 years old or more (n=62)	Total (n=113)
Helminth infection	33(64.7)	34(54.8)	67(59.3)
Ascaris lumbricoides	29(56.9)	26(41.9)	55(48.7)
hookworm	3 (5.9)	15(24.2)	18(15.9)
Trichuris trichiura	3 (5.9)	3 (4.8)	6 (5.3)
Enterobius vermicularis	0 (0)	1 (1.6)	1 (0.9)
Opisthorchis viverrini	1 (2.0)	3 (4.8)	4 (3.5)
Vampirolepis nana	2 (3.6)	2 (2.8)	4 (3.5)
Taenia sp.	0 (0)	1 (1.6)	1 (0.9)
Protozoan infection	24(47.1)	37(59.7)	61(54.0)
Blastcystis hominis	22(43.1)	23(37.1)	45(39.8)
Endolimax nana	4 (7.8)	7(11.2)	11 (9.7)
Chilomastix mesnili	4 (7.8)	5 (8.1)	9 (8.0)
Iodamoeba butschlii	2 (3.9)	6 (9.7)	8 (7.1)
Entamoeba coli	1 (2.0)	6 (9.7)	7 (6.2)
Entamoeba histlytica/E. dispar	1 (2.0)	6 (9.7)	7 (6.2)
Entamoeba hartmani	0 (0)	1 (1.6)	1 (0.9)
Giardia lumbria	1 (2.0)	1 (1.6)	2 (1.8)
Enteromonas hominis	1 (2.0)	0 (0)	1 (0.9)

dispar and *Giardia lambria* infections, however, were not so frequent in the present study.

Among the households in which many families received stool examination, 27 households were selected randomly for the interviews on their life habits (Table 2). It was known that majority of villagers drink boiled water and use tap water to wash cocking materials. Supply of piped water has well known to have preventive effect on soil-transmitted helminthiasis. On the other hand, only 37% of villagers have a toilet in their own houses and more than 35% of the villagers evacuate outside around lake or river. Toilets, whether shared or owned by individual households, seem to be effective for prevention of the parasite infections, however, children still preferred the convenience of defecating in the compound around the house which becomes a source of infection to others. The villagers who always use foot wear were about 30% only. They eat sometimes raw fishes and raw meats as their traditional eating habit. However, food-born parasitic infections, such as *O. viverrini* infection, were extremely few among the villagers.

It had been well documented that a combination of factors such as poor sanitary condition, low socio-economic levels of residents, scarcity of clean water supply and sewage facilities, and ecological and climatic conditions play an important roe in the transmission and high rates of soil-transmitted parasite infections (Fereydoun, 1984; Kan, 1992; Ghani and Oothuman, 1992; Sinniah and

Table 2 The results of questionnaire on living habit of villagers in Toul Roka village, Cambodia

Drinking water	Tap water boild		19(70.3)
	Raining water boild		5(18.5)
	non-boild		3(11.1)
Washing water (cocking material)			
	Tap water		25(92.6)
	Raining water		2 (7.4)
	Well		1 (3.7)
Place of evacuation	Toilet in own house		10(37.0)
	Public toilet		7(25.9)
	Around house		0 (0)
	Around lake or river		6(22.2)
	Others		2 (7.4)
Foot wear	No use	0 (0)	
	Sometime use	19(70.4)	
	Always use sandal	6(22.2)	
	Always use shoes	2 (7.4)	
Eating habit			
	Eat raw fish	No	10(37.0)
		Sometimes	17(63.0)
		Frequently	0 (0)
	Eat raw meat	No	14(51.9)
		Sometimes	13(48.1)
		Frequently	0 (0)
	Eat raw frog	1 (3.7)	
	Eat frog heated	17(62.9)	
	Eat raw snake	0 (0)	
	Eat snake heated	5(18.5)	

Total number of house hold answared the questionnaire were 27.

Rajeswar, 1995). The order of importance of each factor in influencing rate of the parasitic infection is dependent on the level of socio-economic development as a whole and hygienic conditions of the endemic region. The people in areas with a low socio-economic level seem to be busy with their jobs to earn their living; little attention is paid to their personal and family affairs. It has also been shown that the infections are slowly disappearing in areas where good sanitary facilities has been provided and living standards have risen (Pawlowski, 1985). All the above mentioned contributing factors to high prevalence of parasitic infections continue to exist in Cambodia, because of political and social instability due to the civil war over the past decades, and it is expected that the prevalence of intestinal parasitic infection would be high among the inhabitants. However, recent information on the prevalence of parasitic infection is consistently few in this country, although there were some reports on the infection of parasites among Cambodian refugees (Keittivuti *et al.*, 1982; Gyorkos *et al.*, 1992; Lurio *et al.*, 1991; Molina *et al.*, 1988; Parish, 1985; Tittle *et al.*, 1982). In a past survey in a different village in Khampuchea Province, Giboda (1985) has reported that the prevalence of intestinal parasite among 1,739 inhabitants including preschool children was 80%, which was almost the same to the present study. The highest positive rate was obtained in hookworm infection (58.8%) in the past study. On the other hand, the predominant helminth was *A. lumbricoides* (48.7%), followed by hookworm (15.9%), in the present study. One of the characteristic results in the present study was low prevalence of *O. viverrini* infection (3.5%). The liver fluke infection is known to be highly prevalent (more than 50%, in general, in adulthood) in the neighboring Thailand and Laos. *E. histolytica* and *G. lamblia* were predominant protozoan species, with prevalence rates of 16.7% and 14.7%, in the past study. The infection rates of these species, however, were lowered to 6.2% and 1.8% in the present study.

The sanitation improvement and health education were also introduced for transmission control of parasites as an integration program of primary health care. The results of the parasite control program and the reaction toward encouragement on improvement of their living conditions through the control program will be assessed in the following years.

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