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AN ANIMAL MODEL FOR ESTABLISHING CHEMOTHERAPY AGAINST INTRACTABLE TOXOPLASMOSIS IN IMMUNOCOMPROMISED HOSTS BY THE USE OF IFN-γ KNOCKOUT MICE

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ABSTRACT: The *Toxoplasma gondii* number was evaluated by quantitative competitive polymerase chain reaction (QC-PCR) assay with or without sulfamethoxazole treatment in the heart, blood, brain, and small intestine of IFN-γ knockout (GKO) BALB/c (B/c) mice after peroral infection with the cyst-forming Fukaya strain. *T. gondii* infection was observed in the heart, blood, and brain, but not in the small intestine, of mice treated with sulfamethoxazole for 4 weeks. No correlation between *T. gondii* loads and sulfamethoxazole concentrations in tissues and blood was observed. *T. gondii* was not detected in the heart and blood after continuous sulfamethoxazole treatment for two months, but a small number of parasites was demonstrated in the brain. Thus, we successfully established an animal model for evaluating chemotherapy regimens in immunocompromised hosts by using GKO B/c mice infected with *T. gondii*.

Key Words: *Toxoplasma gondii*, immunocompromised hosts, chemotherapy, sulfamethoxazole, IFN-γ knockout mice

INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite that causes life-threatening disease in developing human fetuses and in immunocompromised individuals, particularly patients with acquired immunodeficiency syndrome (AIDS) (Navia et al., 1986). The lack of effective therapy for intractable toxoplasmosis is responsible for the mortality by toxoplasmosis in these patients. In this study, we introduced the GKO mouse as a model of an immunocompromised animal for which to establish an effective chemotherapy protocol for intractable toxoplasmosis. A precise comparative study between *T. gondii* loads and concentrations of sulfamethoxazole was performed in various organs of GKO mice perorally infected with *T. gondii* and treated with sulfamethoxazole.

MATERIALS AND METHODS

Parasites

Cysts of an avirulent Fukaya strain of *T. gondii* (Kamei et al., 1976) were prepared from B10.A(4R) mice that had been infected orally with 5 cysts of Fukaya strain 6 weeks earlier, as previously described (Luo et al., 1995).

Animals

Eight- to twelve-week-old female GKO BALB/c (B/c) mice were genotyped by polymerase chain reaction (PCR) (Tagawa et al., 1997). Age-matched female wild type (WT) B/c mice were purchased from SLC (Hamamatsu, Sizuoka, Japan). GKO mice were classified into two groups after peroral infection with 10 cysts. One group remained untreated and was sacrificed at 4, 7, and 10 days post infection (PI). The second group received sulfamethoxazole continuously from day 4 PI and was sacrificed at 7, 10, 15, 20, and 25 days. WT mice were infected with 10 cysts, and then

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treated with or without sulfamethoxazole.

Drug

Sulfamethoxazole (Shionogi Co., Ltd, Osaka, Japan) was administered in drinking water at a dose of 1 mg/ml. It was first dissolved in 2 N NaOH to facilitate its absorption in water. Then, pH was adjusted to 7 with 10 N HCl. Drug administration was initiated on day 4 PI.

QC- PCR

Using 1μg of genomic DNA each from the heart, brain, blood, and small intestine of the GKO B/c mice, QC- PCR targeting surface antigen 1 (*SAG1*) gene was carried out to determine the distribution of *T. gondii* as described previously (Luo et al., 1997; Kobayashi et al., 1999). The amplified cDNAs were electrophoretically separated on a 1% agarose gel containing ethidium bromide, and the ratio to the subsequently amplified competitor *SAG1* DNA was measured on an IPLab Gel densitometer (Signal Analytical Corp., VA, USA). The abundance of *T. gondii* was calculated as described previously (Luo et al., 1995; 1997).

Treatment of infected mice with exogenous IFN-γ

The treatment of GKO mice with 250 units of recombinant murine (rm) IFN-γ was started 4 days before the infection. The mice were injected intraperitoneally with rmIFN-γ every 2 days for 10 days PI (Mun et al., 2000). The amount of exogenous IFN-γ was comparable with the level of IFN-γ in WT mice infected with *T. gondii* (Suzuki et al., 2000). Parasitic loads were also calculated in the heart and brain at day 10 PI.

Determination of drug concentration

GKO mice were treated with sulfamethoxazole from day 4 to day 25 PI, and the mice were euthanized on days 7, 10, 15, 20, and 25 PI. The concentration of sulfamethoxazole was determined in the blood and tissues (heart, brain, and small intestine) of the mice by high-performance liquid chromatography (HPLC) as previously described (Van de Ven et al., 1995).

Statistics

The significance of differences between groups was determined by Student's *t*- test and that in survival was determined by the Kaplan-Meier method. $P < 0.05$ was considered significant.

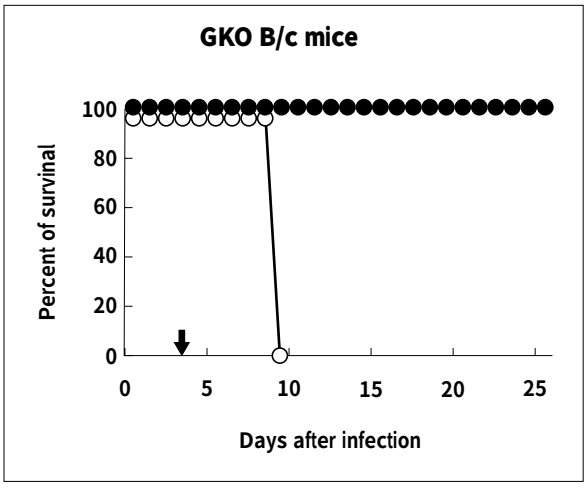

Fig. 1. Sulfamethoxazole treatment of GKO B/c mice challenged perorally with 10 cysts of the Fukaya strain of *T. gondii*. Survival rate was monitored. Six mice were used in each experimental group, and the experiment was repeated three times with similar results. Arrow indicates the starting point of sulfamethoxazole treatment. ○; no treatment, ●; continuous sulfamethoxazole treatment.

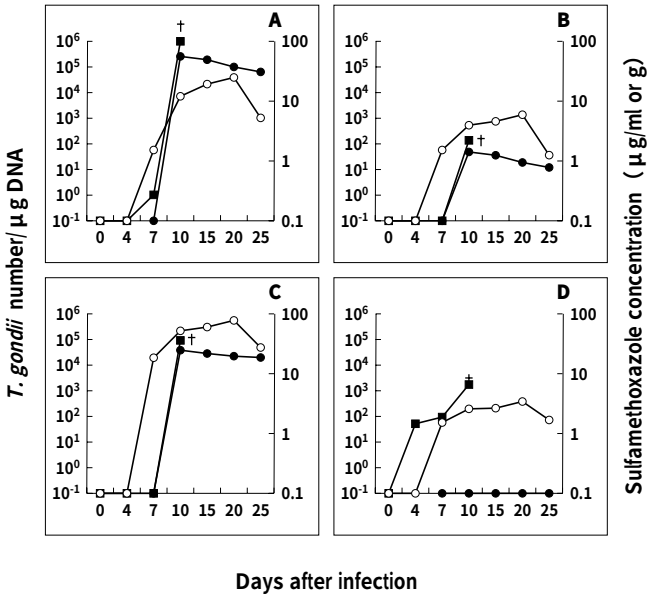

Fig. 2. Effects of sulfamethoxazole treatment on *T. gondii* loads in the organs of GKO B/c mice. *T. gondii* abundance in the heart, brain, blood, and small intestine was estimated by QC-PCR (Fig. 2A, B, C, and D, respectively). Concentration of sulfamethoxazole was expressed as μg/ml in serum and μg/g in organs. Six mice were used in each experimental group and the data shown are representative of three independent experiments with similar results. Data are expressed as the number of parasites per μg of specimen DNA. ■; no treatment, ●; continuous treatment. ○; sulfamethoxazole concentration †; dead mice.

RESULTS AND DISCUSSION

The effects of sulfamethoxazole treatment on the survival rate of GKO mice infected perorally with 10 cysts of the Fukaya strain of *T. gondii* were examined. Untreated mice died within 10 days PI (Fig. 1) but treated mice survived under continuous treatment with the difference in mortality between the two groups being significant ($p < 0.05$). However, only three weeks of sulfamethoxazole treatment failed to eradicate the parasite completely from the heart, brain, and blood (Fig. 2A, B, and C, respectively) although the small intestine became parasite-free during this period (Fig. 2D). Sulfamethoxazole acted only on the rapidly dividing tachyzoites (Lyons et al., 2002). Similar results were reported in previous studies by using sulfadiazine and RH *T. gondii* strain (Dumas et al., 1999; Koppe et al., 1986; Dubey et al., 1998). The tachyzoite is responsible for acute toxoplasmosis (Yano et al., 2002). Our data clearly showed that sulfamethaxazole treatment kept the parasite in the slowly replicating encysted-bradyzoite stage in the heart, brain, and blood of GKO mice, and the present experimental model was physiological regarding the infection route and type of *T. gondii* for the infection.

A high level of sulfamethoxazole concentration was observed in the heart and serum where the parasitic loads were shown to be high (Fig. 2A, and C), while a low level of *T. gondii* loads was observed in the brain where the concentration of sulfamethoxazole was low (Fig. 2B). It should be noted that a detectable level of *T. gondii* was not observed in the small intestine (Fig. 2D) although the level of sulfamethoxazole concentration was similar to that in the brain. These findings revealed that there was no correlation between *T. gondii* loads and sulfamethoxazole concentration.

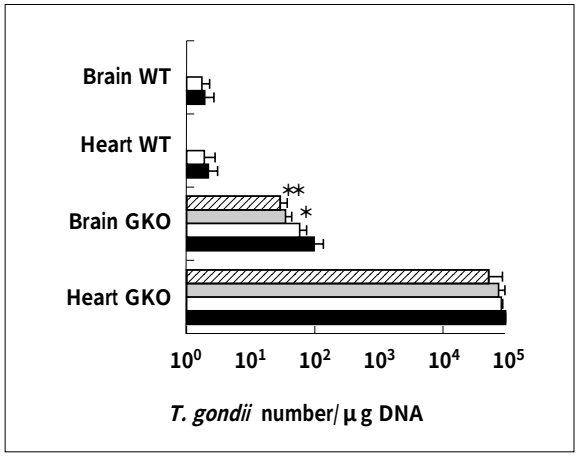

Fig. 3. Effects of intraperitoneal injection of rmIFN-γ alone or with sulfamethoxazole treatment on the parasitic loads in the heart and brain of GKO B/c mice. *T. gondii* loads were assayed 10 days after injection. Three mice were used in each experimental group and the experiment was repeated twice. WT B/c mice were treated with or without sulfamethoxazole. Black bar; mice injected with phosphate buffered saline (PBS), white bar; mice treated with sulfamethoxazole, gray bar; mice injected with rmIFN-γ, white striated bar; mice treated with sulfamethoxazole and rmIFN-γ. *, $P < 0.05$, **, $P < 0.001$.

There were no significant effects of sulfamethoxazole treatment on *T. gondii* loads in the heart and brain of both GKO and WT B/c mice after 10 days P. I (Fig. 3). Furthermore, the treatment with sulfamethoxazole plus exogenous IFN-γ did not reduce the *T. gondii* loads in the heart of GKO mice. This combined treatment decreased the *T. gondii* loads in the brain of GKO mice but the reduction was slight and not comparable with that of WT mice. These results suggest that the treatment of the immunocompromised host with IFN-γ may not be appropriate as a treatment choice against intractable toxoplasmosis.

The present data also clearly showed that the long-duration treatment with sulfamethoxazole for two months eradicated the parasite completely from the blood and heart, but not from the brain where a small number of *T. gondii* remained (Fig. 4). These results indicate that the treatment of immunocompromised hosts with sulfamethoxazole

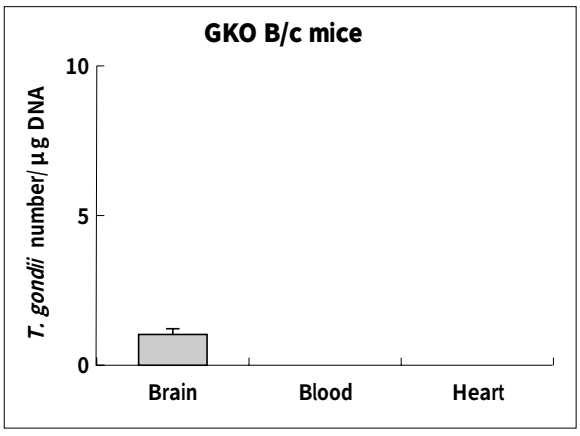

Fig. 4. Influence of long duration (two months) of sulfamethoxazole treatment on down-regulation of parasite loads in the brain, blood, and heart of GKO B/c mice. *T. gondii* abundance was estimated by QC-PCR. Six mice were used in each experimental group and the experiment was repeated three times. Data are expressed as the number of parasites per μg of specimen DNA.

should be continued over the long term. The exact mechanisms by which *T. gondii* loads are decreased in GKO mice with the long-term use of sulfamethoxazole remain to be unraveled. In any case, a mouse model for evaluating chemotherapy regimens in immunocompromised hosts was established by the use of GKO mice.

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DETECTION OF MICROORGANISMS IN TAP WATER IN INDONESIA AND THAILAND

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Abstract: The contamination of tap water by microorganisms was surveyed at Surabaya and Jakarta, Indonesia, and at Hat Yai, Thailand. Water samples were directly collected from house faucets and filtered through membranes. The membranes were examined for protozoan parasites by immunomagnetic separation. Coliform and *Escherichia coli* were examined at each sample collection site using commercially available kits. A total of 115 water samples were examined and 37 (32%) were positive for any of four microorganisms which were two species of protozoa (*Giardia intestinalis* and *Cryptosporidium parvum*) and two kinds of bacteria (coliform and *Escherichia coli*). *G. intestinalis* and *C. parvum* were found in 9% and 1% of total samples, respectively. Of those detected, coliform was the most common and was found in all three areas with a mean detection rate of 30% (15-52%). The water samples that were positive for any of the four types of microorganisms showed a tendency to have lower residual chlorine concentrations and higher turbidities compared with negative samples. It is important to supply safe water in order to maintain people's health because most of the people surveyed (4-88%) ordinarily drank tap water without treating it. Continued efforts are needed to maintain and improve drinking water quality. (193 words)

INTRODUCTION

A safe water supply is advantageous to the health of people. On the other hand, a public water supply will have a serious risk to the health of people if water contamination should occur. Such risks are seen as unexceptional, even in developed countries, where public institutions maintain water quality. Perz *et al*. (1998) made a risk assessment of cryptosporidiosis in New York City, and estimated that the number of tap-water-related cases per annum was 6 to 34. Szewzyk *et al*. (2000) reviewed the microbiological safety of drinking water and described many outbreaks caused by various organisms. The most prominent recorded outbreak occurred in Milwaukee in 1993, involving 403,000 people, with *Cryptosporidium parvum* as the causative agent (Mac Kenzie *et al*., 1994). In addition to this, 703 cases of giardiasis were reported in Massachusetts during 1985 to 1986 (Kent *et al*., 1988), and 6,000 cases in six outbreaks of campylobacteriosis during 1992 to 1996 were reported in Sweden (Szewzyk *et al*., 2000). But in general, as Dawson and Sartory (2000) reported, significant advances in water treatment have taken place over the last century and in developed countries massive improvements have been achieved in the microbiological safety of public drinking water supplies. Incidences of illness due to poor treatment or post-treatment contamination are rare in these countries.

However, in areas with low standards of hygiene and sanitation, the contamination of water by microorganism is still common (Luksamijarulkul *et al*., 1994). In these countries, mainly tropical, it is difficult to maintain water quality at a safe level because of technical and/or economic problems. For example, more than seven species of parasite were recovered from plant-processed drinking water in Argentina (Basualdo *et al*., 2000). In Bangkok, Thailand, 64% of drinking water sources tested were contaminated with coliform (Luksamijarulkul *et al*., 1994). In Taiwan, 39% to 77% of treated water samples were positive for *Giardia* sp. and *Cryptosporidium* sp. (Hsu *et al*., 1999). It appears that the health hazard posed to people who are supplied such low quality water is significant. In spite of such

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circumstances, little is known about how frequent tap-water is contaminated with microorganisms and how frequent tap water is used in developing countries.

This paper reports the results of a field survey done in Indonesia and Thailand to clarify the relationship between tap water quality and contamination by microorganism. Information regarding tap water usage was obtained through questionnaire surveys and the results were analyzed.

MATERIALS AND METHODS

1. Survey areas and period

Surveys were conducted from August 2000 to August 2001 in Surabaya and Jakarta, Indonesia, and in Hat Yai, Thailand. Surabaya is the second largest city in Indonesia and is located in the east of Java Island. Jakarta, located in the west of Java Island, is the capital city of Indonesia containing many modern buildings and has a population of 9.1 million. Hat Yai is a city in the south of Thailand near the Thai border with Malaysia. Questionnaire surveys were performed at health centers in each area with the cooperation of staff members.

2. Filtration of sample water

Filtration of tap water was performed for *C. parvum* oocysts and *Giardia intestinalis* cyst [oocysts of *C. parvum* and cysts of *G. intestinalis* are expressed as (oo)cysts]. Sample water was collected directly from a faucet in each house visited. Permission to collect water and confirmation of its origin were recorded. A polycarbonate membrane (142 mm in diameter, 3 μm pore size; Nucleopore, Whatman, USA) was inserted in a filter folder (Advantec KS-142, Tokyo, Japan), and 5 to 50 liters of tap water were filtered from the pipe that was connected directly to a faucet. To estimate the total filtered volume, the filtered water was kept in a bucket. We did not use any pressure device for the filtration, so we were only able to filter a small amount of tap water when the water pressure was low, or when turbidity was high. A filter membrane was put into each of the 50 ml centrifuge tubes that contained 30-40 ml of 1% formalin, kept at room temperature, and transported to Japan.

3. Parasitological measurements

In Japan, each membrane was transferred to a 200 ml beaker containing 100 ml of PBST (PBS with 0.1% of Tween 80), and (oo)cysts on the membrane were eluted by agitation for 2 min by hand and sonication for 2 min using a sonicator (IUC-7321, Tocho co., Tokyo, Japan). The (oo) cyst suspension was again filtered using a polycarbonate membrane (90 mm in diameter and 3 μm pore size) and transferred to a 10 ml test tube (Dynal, Oslo, Norway) containing 10 ml PBS. The membrane was again treated by sonication and then removed. The remaining solution was further processed by an immunomagnetic separation method (Dynal). The purified and concentrated solution was dried on a slide glass and stained with anti-*Cryptosporidium*/*Giardia* monoclonal antibodies conjugated with fluorescein (Aqua G/C Direct, Waterborne, LA, USA) for 30 min and 6-diamidino-2-phenylindole (DAPI) for 2 min, and the (oo)cysts were then observed under a fluorescent microscope (400- to 440- nm filter in the former, and 330- to 385- nm filter in the latter).

4. Bacterial measurements

Tap water samples were examined for coliform and *Escherichia coli* using a commercially available kit (Colilert, IDEXX, Maine, USA) at the sample collection site. This kit allowed the simultaneous qualitative detection of coliform and *E. coli*.

5. Water quality measurements

Water quality was examined simultaneously when each sample was collected. Residual chlorine was measured with the Lovibond 2000 kit (Tintometer Limited, England), and turbidity was measured using a turbidity meter (2100P, Hach Co., Colorado, USA).

6. Questionnaire survey

A questionnaire survey was performed in three areas to investigate number of households equipped with tap water and the frequency of drinking of tap water by local people. Following our comprehensive explanation of the study and the inhabitants' consent, we surveyed them on seven items including whether tap water was available in the house, or what type of drinking water was usually used.

RESULTS

1. Contamination with microorganisms

A total of 115 water samples were examined and 37 (32%) were positive for microorganisms. These microorganisms were two kinds of bacteria (coliform and *E. coli*) and two species of protozoa (*G. intestinalis* and *C. parvum*). Of the microorganisms detected, coliform was the most common and was found in all three areas with a mean detection rate of 30% (15-52%). *E. coli* was found with a mean of 10% (0-23%). Among protozoa, *G. intestinalis* was found with a mean of 9% (0-25%) (Table 1). The mean number of cysts found in 10 positive samples was 2.5 (1-8). One *C. parvum* oocyst was found in one sample (S-1) in Surabaya (Tables 1 and 2). The intensity of contamination in the three areas was compared. In Surabaya, which

Table 1 Contamination of tap water with microorganisms

Locality	No. of samp.	No. of +ve (%)	No. of -ve (%)	Contamination with:* Bacteria Coli.	Bacteria E. c	Protozoa G. i	Protozoa C. p	Water quality Mean Cl_2 †	Mean NTU ‡
Surabaya	40	23(58)	-	21(52)	9(23)	10(25)	1(3)	0.08	0.72
		-	17(42)					0.19	0.67
Jakarta	27	4(15)	-	4(15)	0(0)	0(0)	0(0)	0.05	0.56
		-	23(85)					0.54	0.38
Hat Yai	48	10(21)	-	10(20)	2(4)	0(0)	0(0)	0.06	1.18
		-	38(79)					0.28	1.07
Total	115	37(32)	78(68)	35(30)	11(10)	10(9)	1(1)	0.25	0.79

* E. c: *Escherichia coli*, G. i: *Giardia intestinalis*, C. p: *Cryptosporidium parvum*.
† Less than 0.1 mg/ml is tentatively considered as 0.05 mg/ml and calculated.
‡ National Turbidity Unit.

Table 2 Relationship between microorganism contamination and tap water quality

Sample* nos.	Water filtered (L)	Contaminated with: † Coli.	E. c	G. i	C. p	Quality of water Cl_2	NTU ‡
S-1	70	+	-	+(2)	+(1)	<0.1	0.39
S-2	55	+	-	-	-	<0.1	0.44
S-3	22	+	-	-	-	<0.1	0.46
S-4	25	+	-	-	-	<0.1	0.92
S-5	20	+	-	-	-	<0.1	1.14
S-6	20	+	-	+(1)	-	<0.1	0.63
S-7	14	-	-	+(1)	-	0.6	0.83
S-8	15	-	-	+(4)	-	<0.1	1.06
S-9	30	+	+	+(2)	-	<0.1	0.36
S-10	ND	+	+	ND	ND	<0.1	1.85
S-11	ND	+	+	ND	ND	<0.1	0.38
S-12	ND	+	+	ND	ND	<0.1	0.57
S-13	ND	+	-	ND	ND	<0.1	0.44
S-14	25	+	-	+(3)	-	<0.1	0.56
S-15	40	+	-	-	-	<0.1	0.53
S-16	30	+	+	-	-	<0.1	0.65
S-17	30	+	-	+(1)	-	<0.1	0.57
S-18	40	+	+	+(8)	-	<0.1	0.48
S-19	30	+	-	-	-	<0.1	0.6
S-20	8	+	-	-	-	<0.1	0.56
S-21	ND	+	+	ND	ND	<0.1	1.28
S-22	50	+	+	+(2)	-	<0.1	0.61
S-23	30	+	+	+(1)	-	<0.1	0.93
J-1	ND	+	-	ND	ND	<0.1	0.4
J-2	15	+	-	ND	ND	<0.1	0.52
J-3	50	+	-	-	-	<0.1	0.92
J-4	40	+	-	-	-	<0.1	0.4
H-1	50	+	-	-	-	<0.1	3.13
H-2	50	+	-	-	-	<0.1	0.66
H-3	50	+	+	-	-	<0.1	1.36
H-4	50	+	-	-	-	<0.1	0.86
H-5	50	+	-	-	-	0.1	1.72
H-6	50	+	+	-	-	<0.1	1.57
H-7	50	+	-	-	-	<0.1	0.85
H-8	50	+	-	-	-	<0.1	0.71
H-9	50	+	-	-	-	<0.1	0.53
H-10	50	+	-	-	-	<0.1	0.94

*Samples were collected from Surabaya (S), Jakarta (J), and Hat Yai (H).
† Coli.: coliform, E. c: *Escherichia coli*, G. i: *Giardia intestinalis*, C. p: *Cryptosporidium parvum*.
‡ National Turbidity Unit.

showed the highest contamination rate, 58% of tap water samples were positive for microorganisms, and all of the four types of microorganisms recovered through this study were detected (Table 1). In Jakarta 15% of water samples were positive for coliform, and in Hat Yai 20% and 4% were positive for coliform and *E. coli*, respectively (Table 1).

2. Microorganism contamination and water quality

Table 2 shows the details of 37 samples that had any of four microorganisms: 23 samples from Surabaya (S), 4 from Jakarta (J), and 10 from Hat Yai (H).

Bacterial contamination was closely related to the concentration of residual chlorine but protozoan contamination was not. Of the 35 bacteria-positive samples, only one (3%, H-5) had 0.1 mg/l of residual chlorine and the remaining 34 (97%) had a concentration of less than 0.1mg/l. Of the 115 water samples examined, 66 (57%) had less than 0.1mg/l of residual chlorine concentration; of those, 53% were positive for bacteria. In contrast to this *G. intestinalis*, although only a single sample, was found even in a residual chlorine concentration of 0.6 mg/l. Five positive samples from Surabaya (S-1, S-9, S-18, S-22, S-23) were contaminated with three kinds of microorganisms (Table 2).

When the positive and the negative samples were compared using the parameters of residual chlorine concentration and turbidity, the former had a tendency to have lower residual chlorine concentrations and had higher turbidities than the latter (Table1).

3. Questionnaire survey

One of the aims of this survey was to confirm whether the local people used tap water as a source of drinking water. A total of 352 questionnaires were completed and collected in the three areas examined, and revealed that 80% of the people used tap water. Of the people who had tap water, more than half (54%) drank it without boiling or

Table 3 Utility of water by people in three different areas as revealed by questionnaire survey

Locality	No. of questionnaires recovered	With water supply system			Without water supply system	
		No. (%)	Drink tap water without boiling/filtering (%)	Sometimes we drink: (%)	No. (%)	Sometimes we drink: (%)
Jakarta (Indonesia)	100	100 (100)	67 (67)	bottled water (20) well water (7)	0 (0)	
Surabaya (Indonesia)	102	91 (89)	80 (88)	bottled water (31) well water (1)	11 (11)	well water (55) bottled water (36)
Hat Yai (Thailand)	150	90 (60)	4 (4)	bottled water (61) well water (18)	60 (40)	well water (40) bottled water (13)
Total	352	281 (80)	151 (54)	Bottled and well water	71 (20)	Well and bottled water

filtering it. This rate varied from area to area: 88% in Surabaya but only 4% in Hat Yai. Those who did not have tap water to the house used bottled water for drinking, but at the same time, 40-55% of them also used well water (Table 3).

DISCUSSION

A safe water supply system is a "double-edged sword". If this system functions well, it can maintain the health of a population. But if not, large public health problems occur (Dawson and Sartory, 2000; Szewzyk *et al*., 2000). In both developed countries where urbanization or centralization of the population has progressed, and developing countries where water quality is not maintained, such apprehensions are present. Unfortunately, the water quality found in this study was poor. As Steiner *et al*. (1997) stated, the leading cause of infant mortality in the developing world is infectious diarrhea, and the prevalence of the diarrhea pathogen is largely influenced by the quality of clean water available for drinking. Recent guidelines and legislation of the European Union Council Directive 98/83/EC and the World Health Organization stated that drinking water should be safe and not contain pathogenic microorganisms, or contain only such low numbers that the risk for acquiring waterborne infections is below an acceptable limit (Szewzyk *et al*., 2000).

Four kinds of microorganisms were detected throughout the survey. Most of these microorganisms have an animal reservoir from which they are transmitted to humans, directly or via the environment. These microorganisms are resistant to environmental stress and have a very low infective dose. It appears that even low-level contamination of water supply systems may lead to infections and disease in the exposed population (Szewzyk *et al*., 2000).

Emerging and reemerging infectious parasites in drinking water have become increasingly important during the last few decades. These include newly recognized protozoa such as *C. parvum* and *Cyclospora cayetanensis*, or *G. intestinalis* known as traveler's diarrhea (Rose and Slifko, 1999). Since *C. parvum* and *G. intestinalis* have a tolerance to chlorine, they sometimes become causative agents of waterborne infectious diseases. Among these protozoa, the detection of *C. parvum* oocysts from water samples is very difficult because the oocyst is small, has little morphological character and, more importantly, is only seen in small numbers in water samples. In the United States and Britain, methods for detecting oocysts from water have been reported (Fricker and Crabb, 1998). The method we developed for this survey, which is simple and highly efficient in recovering oocysts, is suitable for use in developing countries. A notable feature of this method is that it does not involve a centrifugation process during isolation because we experienced that centrifugation reduced the recovery rate.

E. coli is a bacterial indicator of faecal contamination. In recent years, enterococci, faecal bacteria of the genera *Enterococcus* and *Streptococcus*, have also been given significant recognition as faecal indicators (Dawson and Sartory, 2000), though we did not examine them. It is reported that 0.2 mg/l of residual chlorine can kill 90% of *E. coli* within two hours (Hsu *et al*., 1984). We suspect that the shortage of residual chlorine concentration was one cause for the bacterial contamination of tap water. There are many factors contributing to the decline of residual chlorine concentration in tap water. In this survey, the reason of low chlorine concentrations may be the insufficient removal of organic matter at waterworks, based on relatively high turbidities of tap water and river water from which the tap water is derived. When flood damaged Hat Yai City in 2000, we revealed that the mean residual chlorine concentration in 30 tap water samples was maintained at 0.5 mg/l (data not shown). That is, the public works adjustment of the chlorine concentration according to the situation occurred. However, during the usual operation of the water works in the same city in 2001, 0.1 mg/l or less chlorine concentration was frequently observed. Therefore, we cannot deny

the possibility of mishandled operations at the water purification plant.

Of the three areas studied, Surabaya City had the lowest water quality. Despite this, 88% of people drank tap water without treating it. As a primary prevention, an immediate improvement of the water-purifying environment, as well as the education of the populace, are needed. The spread rate of water supply system in Hat Yai was the lowest (60%). But at the same time, the number of the people who drank tap water without treating it was the lowest. We thought that this was probably due to knowledge accumulated through the experience of the local people.

Poorly managed public water supplies have the potential to make a large number of people ill (Dawson and Sartory, 2000). Therefore, we have to continue to put efforts in supplying of safe drinking water and to educate the people who use it.

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A RAPID SINGLE-STEP SCREENING METHOD FOR GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY IN FIELD APPLICATIONS

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Abstract. The single-step screening method (SSS) is a qualitative rapid screening test for glucose-6-phosphate dehydrogenase (G6PD) deficiency based on blue formazan formation on anion-exchanger. The reaction mixture contains equal volumes of anion exchanger, substrate mixture, coloring mixture (MTT-PMS mix) and distilled water. We assessed the stability of the reaction mixture and evaluated its reliability with two anion exchangers, DEAE-Sephadex A-50™ and DEAE-Sephacel™, for applications in tests under field conditions. The reaction mixture was sufficiently stable under conditions of incubation at 70℃ for 6 hours or vigorous shaking for 24 hours at room temperature. The reaction mixture could be kept at 30-35℃ for 14 days under indoor conditions without shielding if it contained no MTT-PMS mix. The coloring was detectable even in diluted blood with hemoglobin concentration as low as 1.6 g/dl. Under laboratory conditions, the proportion of the samples with 10% of the normal level of activity that were diagnosed as 'low activity' was higher with DEAE-Sephacel (92%) than with DEAE-Sephadex A-50 (81%) ($p=0.023$). The proportion of the samples with normal activity that were diagnosed as 'normal' was 98% with DEAE-Sephacel and 100% with DEAE-Sephadex A-50. In field samples obtained in Myanmar and Indonesia, the sensitivity was lower ($P=0.03$ using DEAE-Sephadex and $p< 0.001$using DEAE-Sephacel) when we used the blue formazan spot test (BFST) as the standard. Twenty-three of 27 G6PD-deficient individuals subjected to genetic analysis were found to have mutations. All individuals who had concordant results between the SSS and the blue formazan spot test (BFST) carried molecular mutations. One case of G6PD mutation was detected among four cases diagnosed as G6PD-deficient by SSS with DEAE-Sephacel™, but diagnosed as 'normal' by BFST. The costs of one test with the DEAE-Sephadex A-50™ and the DEAE-Sephacel™ system were 0.15 US dollar and 0.30 US dollar, respectively. (297 words)

Key words: Single-step screening test, Glucose-6-phosphate dehydrogenase deficiency, Device approval, Field trial, Malaria

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is a frequent and heterogeneous X-chromosome-linked enzyme abnormality. As G6PD plays a key role in maintaining erythrocytes, G6PD deficiency possibly results in acute hemolysis after exposure to various oxidative stresses, including infections, medications, and fava beans (favism). A striking correlation is observed between the prevalence of G6PD deficiency and historical malaria endemicity, particularly in tropical and sub-tropical areas (W. H.O. Working Group, 1989). Therefore, it is important to detect and inform G6PD-deficient individuals in and from areas in which malaria is endemic before exposing such individuals to oxidative stress in order to avoid acute hemolytic attack, especially hemolytic attack caused by primaquine.

For field screening of G6PD deficiency, the test used should be simple to perform and affordable. It is also advantageous if the test reagents can be stored and the reaction can be carried out at around room temperature, particularly in areas with an insufficient supply of electricity.

Several tests, including the G6PD spot test (Fairbanks and Beutler, 1962), fluorescent spot test (Beutler, 1966), and blue formazan spot test (Fujii *et al*., 1984) have been developed for field screening of G6PD deficiency; however most of them are complicated or expensive. One of the tests, the blue formazan spot test (BFST) (Fujii *et al*., 1984; Pujades *et al*., 1999), has been used for field studies because of its comparatively simple procedure and sufficiently

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low cost. However, BFST requires 8 hours of incubation for diagnosis, which makes immediate on-site diagnosis difficult.

Recently, a simple and quick screening method, a single-step screening test (SSS) was developed (Hirono *et al*., 1998). Our previous trials showed the usefulness of SSS in field studies (Tantular *et al*., 1999), but the stability and reliability of the test have not been fully assessed. In this study, we assessed in detail the stability of SSS and the results of the reliability of SSS use under field conditions.

MATERIALS AND METHODS

SSS is a qualitative test that assesses the formation of blue formazan along with the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) by G6PD absorbed on an anion exchanger. The reaction mixture consists of equal volumes (200 μl each) of substrate mixture containing 5 mM glucose-6-phosphate (G6P) and 0.4 mM oxidized nicotinamide adenine dinucleotide phosphate (NADP), coloring mixture (MTT-PMS mix) containing 0.025% 3 (4,5 dimethylthiazolyl 1-2) 2,5 diphenyltetrazolium bromide (MTT), 0.025% phenazine methosulphate (PMS), anion exchanger (originally DEAE-Sephadex A-50™) and distilled water. The test can detect G6PD-deficient individuals with less than 10% residual activity within 40 minutes without any special equipment.

The procedures and chemicals used for SSS (Hirono *et al*., 1998) and BFST (Fujii *et al*., 1989) were as described, with slight modifications in the procedure for SSS: 0.05% sodium azide (NaN_3) was added and saponin was omitted in the reaction mixture to avoid the growth of microorganisms. We assessed the reliability of two different anion exchangers in SSS: translucent DEAE-Sephadex A-50™ and white-colored DEAE-Sephacel™ (Amersham Pharmacia Biotech, Buckinghamshire, UK). When using the DEAE-Sephacel™ system, 4.8mM of oxidative glutathione (GSSG) was added to optimize the reactivity (Hirono *et al*., 1998).

The blood samples for laboratory assessment were drawn from one of the authors who was confirmed phenotypically and genotypically to have normal G6PD. The G6PD gene was analyzed by direct sequencing of all coding exons. The G6PD-deficient blood was artificially made from the blood of the same volunteer by heat inactivation at 56℃ for 15 minutes and proved to have no residual G6PD activity. Nine volumes of heat-inactivated blood and one volume of fresh blood were mixed to make G6PD-deficient samples with 10% of the normal activity. The samples for field comparison and genetic analysis were obtained from local residents who participated in an extensive field study with the aim of quick detection of malaria and G6PD deficiency (Tantular *et al*. 1999) in the Taninthayi Division in Myanmar and on Buru Island in Indonesia, 1998. Prior to the study, the outline and procedures of this research were discussed within the committees of the national and local governments in Indonesia and Myanmar. The participants were orally informed of and gave consent to the examination twice before screening and blood drawing. We did not obtain written informed consent from each participant because of the opinion of local co-organizers about the participants' literacy and differences of local culture. The field samples were used to assess the sensitivity and specificity of SSS using BFST as the standard. We also compared the 'G6PD-deficient' samples of SSS with BFST, using genetic analysis as the standard.

To assess the stability of the test mixtures of SSS under various laboratory conditions, we simulated three different conditions which may often occur during field studies in tropical areas: 30-35℃ for 14 days (storage at room temperature), 70℃ for 6 hours in an oven (being left in a car), continuous shaking using a shaker (TAITEC R-1) at maximum strength (200 rotations/minute) for 24 hours at room temperature (transporting over a rough surface). We also examined the photosensitivity of the test, because the dissolved MTT-PMS mixture is highly photosensitive (Fairbanks and Beutler, 1962). For simulating anemia, which is a common illness and may affect the results (Fairbanks and Beutler, 1962), serial two-fold dilutions of blood with normal G6PD activity from the same volunteer in other laboratory tests were made with phosphate buffered saline (PBS) to one-eighth of the original concentration (the estimated hemoglobin concentration was then 1.6 g/dl). For all of the assessments, the combination of newly prepared testing mixture and fresh blood was used for the standard of the normal reaction.

For the evaluation of reliability, the results were categorized into two groups, 'low activity' and 'normal activity', based on color development of the anion-exchanger. Two investigators who were blinded to the actual activity of samples then evaluated the enzyme activity (50 deficient and 50 normal per person) independently as 'low activity' or 'normal activity'. The results were analyzed by the chi-square test using a 2×2 table or Fisher's exact test. P values less than 0.05 were considered as statistically significant.

BFST was used as the standard for field assessment of the sensitivity and specificity of SSS. The SSS was performed at each field site and samples for BFST were obtained on cation-exchange paper (Whatman P-81) (Fujii *et al*., 1984) at the same time and dried. The BFST was performed within the same day, and thus the results were obtained the next day. The results were analyzed statistically as described above.

For comparison of the results of SSS and BFST with the results of genetic analysis, the participants were screened by SSS, and a blood spot was obtained in the same way as described above. The participants were diagnosed with G6PD deficiency based on the results of SSS, and their blood was drawn for genetic analysis. The procedures for genetic analysis were described previously (Hirono *et al*., 1994; Hirono *et al*., 1997, Iwai *et al*., 2001).

RESULTS AND DISCUSSION

The reaction mixture of SSS retained sufficient reactivity compared with the control mixture after exposure to 70 ℃ for 6 hours or continuous shaking for 24 hours at room temperature. Reaction mixture without the MTT-PMS mix could be stored at 30-35 ℃ for 14 days under indoor conditions without shielding. These features would make this test appropriate for regular use in primary care and in field studies in rural areas in developing countries. A previously reported 1-year storage trial (Hirono *et al*., 1998) showed that DEAE-Sephadex™ can be kept at room temperature, the MTT-PMS mix can be stored at 4 ℃, and the substrate mixture is stable at −20 ℃. If a refrigerator and freezer are available, each mixture can be safely stored for at least1year.

In our simulation of anemia, the reaction mixture yielded detectable color development when the estimated hemoglobin concentration of the G6PD-normal blood was as low as 1.6 g/dl. Because elevated levels of reticulocytes lead to higher activity of peripheral blood G6PD in the anemic condition (Jansen *et al*., 1985), our finding implies that the G6PD-normal samples of patients with severe anemia should not be frequently misdiagnosed as 'G6PD-deficient'.

Table 1 shows the reliability of the 2 anion-exchangers. Under laboratory conditions, a higher proportion of the samples with 10% of normal activity were diagnosed as 'low activity' with DEAE-Sephacel™ (92%) than with DEAE-Sephadex A-50™ (81%) (p=0.023). With the DEAE-Sephadex A-50™ system, nearly 20% of G6PD-deficient samples were judged as normal. The red-colored hemoglobin in the reaction mixture together with the translucent DEAE-Sephadex A-50™ particles may cause misdiagnosis. The proportion of the samples with normal activity that were diagnosed as 'normal' was 98% with DEAE-Sephacel™ and 100% with DEAE-Sephadex A-50™.

The sensitivity and specificity in field tests are shown in Table 2. We used BFST as the standard because a previous study using the samples of genetically diagnosed G6PD-deficient patients showed that BFST had 100% sensitivity in hemizygotes and 75% in heterozygotes and 100% specificity (Pujades *et al*., 1999). The sensitivity was significantly higher in males than in females when we use DEAE-Sephacel as anion-exchanger (P=0.04). Neither the sensitivity nor specificity differed significantly between DEAE-Sephadex™ and DEAE-Sephacel™.

Comparison of field testing with laboratory assessment revealed that the sensitivity of field testing was lower than that of laboratory assessment. The P-value was 0.03 using DEAE-Sephadex A-50 and less than 0.001 using DEAE-Sephacel. We found no difference in specificity. In field assessment, the sensitivity was significantly higher among males when we used DEAE-Sephacel (p=0.04). The actual value of the sensitivity was also high among males tested with DEAE-Sephacel, although there was no significant difference (p=0.12). We suppose the discrepancy between laboratory and field assessments may be due to 3 reasons. One is the difference of genetic status between males and females. Because G6PD deficiency is an X-chromosome-linked abnormality, the enzyme activity is particularly vari-

Table 1. Laboratory evaluation of the single-step screening method (SSS) with 2 anion exchangers

A. DEAE-Sephadex A-50

Diagnosis by SSS	Residual activity of G6PD		Total
	10%*	100%	
Low activity	81	0	81
Normal activity	19	100	119
Total	100	100	200

B. DEAE-Sephacel

Diagnosis by SSS	Residual activity of G6PD		Total
	10%*	100%	
Low activity	92	2	94
Normal activity	8	98	106
Total	100	100	200

*The proportion is significantly different between the DEAE-Sephadex A-50 system and DEAE-Sephacel system

Table 2. Sensitivity and specificity of the SSS using BFST as a standard

A. DEAE-Sephadex A-50

Diagnosis by BFST	Diagnosis by SSS (male/female)		Total (male/female)
	G6PD deficiency	G6PD- normal	
G6PD deficiency	20(18/2)	12(8/4)	32(26/6)
G6PD normal	3(2/1)	187(96/91)	190(98/92)
Total	23(20/3)	199(104/95)	222(124/98)

Sensitivity: male 69% (18/26), female 33% (2/6)
Specificity:male 98% (96/98), female 99% (91/92)

B. DEAE-Sephacel

Diagnosis by BFST	Diagnosis by SSS (male/female)		Total (male/female)
	G6PD deficiency	G6PD- normal	
G6PD deficiency	17(13/4)	9(3/6)	26(16/10)
G6PD normal	9(5/4)	354(171/183)	363(176/187)
Total	26(18/8)	363(174/189)	389(192/197)

Sensitivity: male 81% (13/16), female 40% (4/10)*
Specificity:male 97% (171/176), female 98% (183/187)
*Significantly different (P=0.04)

able in heterozygous females.

The crude G6PD level of blood may also be affected by anemia caused by various illnesses. Variable Hb levels together with various levels of G6PD activity in field samples could partially explain the discrepancy of these 2 tests, although our laboratory assessment showed that the G6PD-deficient samples could be distinguished from G6PD-normal samples.

We also consider it possible that the discrepancy between two tests may be due to inaccurate evaluation by SSS. The definite cut-off level of coloring should be assessed by comparison of BFST and SSS using genetically confirmed samples.

Comparison of the results of SSS and BFST with the results of genetic analysis showed that 23 of 27 G6PD-deficient individuals detected by SSS, including two heterozygous females, had mutations (Table 3). All samples for which the results of SSS coincided with those of BFST were shown to have mutations, regardless of the anion exchanger used for the analysis. One case of class 2 G6PD mutation, 383 T→C (G6PD Vanua Lava), was detected among four cases that were diagnosed as 'G6PD-deficient' using SSS with DEAE-Sephacel™ but diagnosed as 'G6PD normal' with BFST. This result suggests that some cases of G6PD deficiency may be undetectable using BFST, although a study showed that the G6PD-deficient hemizygotes could be distinguished from heterozygotes and normal controls using BFST (Pujades *et al*., 1999).

Including all reagents and disposable supplies, the estimated cost for one test of SSS with DEAE-Sephadex A-50™ was approximately half that of a test of SSS with the DEAE-Sephacel™ (0.15 US dollar and 0.30 US dollar, respectively). The cost of both of these systems is below an acceptable limit.

SSS is a quick, simple and reliable screening test for G6PD deficiency, although a larger field study is necessary for more precise evaluation of SSS. All the procedures in this test could be completed in field conditions without any electric equipment except during some preparations prior to the study. Using either anion exchanger, it can be used for quick detection of G6PD deficiency in various situations including field surveys, mass-treatment of malaria and differential diagnosis of hemolytic anemia, particularly in areas with substandard laboratory conditions. Although the white-colored DEAE-Sephacel™ gives better reaction visibility, the DEAE-Sephadex A-50™ system is more cost-effective, and is therefore preferable financially, particularly for field studies.

Table 3. The results of genetic analysis and the BFST of 'G6PD-deficient' samples diagnosed by SSS

Diagosis by BFST	DEAE-Sephadex A-50		DEAE-Sephacel	
	Analyzed	Variants	Analyzed	Variants
Deficient	17	17(1)	5	5(1)
Normal	1	0	4	1
Total	18	17(1)	9	6(1)

Heterozygotes in parentheses

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Short communication

NATURAL INFECTIONS WITH FILARIAL LARVAE IN TWO SPECIES OF BLACK FLIES (DIPTERA: SIMULIIDAE) IN NORTHERN THAILAND

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Abstract: To find out the natural infection with filarial larvae, female adult black flies were collected on a human attractant in December, 2001 at Tambol Ban Laung (altitude 750 m), Doi Inthanon National Park, in northern Thailand. The total number of females collected was 823: of which 557 (67.7%) were identified as *Simulium asakoae* Takaoka et Davies, 144 (17.5%) as *S. nigrogilvum* Summers, 97 (11.8%) as *S. nakhonense* Takaoka et Suzuki and 25 (3%) as other six simuliid species. By dissections, eight third- and one second-stage larvae of unidentified filarial species were found in one of 138 *S. nigrogilvum* and one of 484 *S. asakoae*, respectively. Non-filarial nematodes were found in 1.03% (5/484) of *S. asakoae.* This is the first report of natural infections of two black-fly species, *S. nigrogilvum* and *S. asakoae*, with a filarial larva.

Key words: black fly, filaria, natural infection, *Onchocerca*, Simuliidae, Thailand

INTRODUCTION

Simuliidae or black flies have been well known to be a pest of humans and animals and also vectors of some parasites and pathogens (Crosskey, 1990). The main medical significance is the transmission of *Onchocerca volvulus* to humans by the bites of the flies in Africa and Central and South America; certain simuliid species are suspected to be a probable vector of zoonotic onchocerciasis which occurs sporadically in Japan, North America and Europe (e.g., Hashimoto *et al.*, 1990; Takaoka *et al.*, 1996).

In the Oriental Region, several man-biting simuliid species have been reported, e.g., *Simulium asishi* Datta, *S. himalayense* Puri, *S. indicum* Becher, *S. japonicum* Matsumura, *S. nodosum* Puri and *S. tenuistylum* Datta (Datta, 1992; Lewis, 1974; Takaoka, 1977). Several other species have been known as a pest of animals (Datta, 1992; Datta and Dasgupta, 1975; Friederichs, 1925). Recently, we examined adult female black flies captured on humans and water buffalos at Ban Pang Fan (250 m in altitude), Chiang Mai Province, in northern Thailand, and reported for the first time the natural infections of *S. nodosum* with *Onchocerca* larvae (Takaoka *et al*., 2003).

To get further information on natural filarial infections of black flies in northern Thailand, we collected adult black flies at Tambol Ban Laung (750 m in altitude) in Chiang Mai Province. Here we report two more black-fly species naturally infected with a filarial larva.

MATERIALS AND METHODS

Study area

Adult black flies were collected at a site exposed to the sun in the village of Tambol Ban Laung (18 °25'-18 °37' N and 98 °27'-98 °42' E: ca. 750 m in altitude), Doi Inthanon National Park, Chiang Mai Province, northern Thailand.

Collection of adult black flies

The collection was made on 16 December of 2001, for 12 hr from 06.00 to 18.00 hours using a human attractant (WC, one of the authors) with his legs below the knees exposed. Female black flies landing on or flighting around the

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attractant were caught by an insect net, killed and preserved in a vial with 70% ethanol solution. Air temperature was recorded every hour from 06.00 to 18.00.

Dissections of adult black flies for filarial larvae

After classification to species using the descriptions of Puri (1933) and Takaoka and Suzuki (1984), most of flies were microscopically dissected and searched for filarial larvae in a drop of 0.5% Giemsa's stain solution on a slide glass. The filarial larvae found were preserved in 2% formalin solution for morphometric observations. Generic diagnosis of third-stage and second-stage filarial larvae followed Bain and Chabaud (1986).

RESULTS

Black-fly species collected

The air temperature measured fluctuated between 10.7 ℃ and 19.8 ℃. A total of 823 females of nine black-fly species was collected on a human attractant. The most abundant species was *S. asakoae* Takaoka et Davies (67.7%) followed by *S. nigrogilvum* Summers (17.5%) and *S. nakhonense* Takaoka et Suzuki (11.8%), and six other species were few, consisting of 0.1-2.2% (Table 1).

Table 1. Numbers of female adult black flies collected on a human, at Tambol Ban Laung, Doi Inthanon National Park, Chiang Mai Province, northern Thailand, on December 16, 2001.

Simulium spp.	No. of collected	% of total
S. asakoae	557	67.7
S. nigrogilvum	144	17.5
S. nakhonense	97	11.8
S. chamlongi	18	3.0
S. fenestratum	2	
S. nodosum	2	
S. chumpornense	1	
S. siamense	1	
S. sp. E	1	
Total	823	100.0

Natural infections with filarial larvae

Natural filarial infections were found in 0.72% (1/138) of *S. nigrogilvum* and 0.21% (1/484) of *S. asakoae.* No infection was found in 97 *S. nakhonense* dissected. Six other minor species were not dissected. The infected *S. nigrogilvum* harboured eight third-stage larvae (L_3) in the thorax. Among them, four larvae measured were 800-900 μm long by 25-31 μm wide (Fig. 1A). The length ratio of the esophagus against the whole body of the three larvae was 0.37-0.41. The L_3 had a relatively short and somewhat conical tail with a salient axial point (Fig. 1B). The other infected fly *S. asakoae* harboured one second-stage filarial larva (L_2) in the thorax. The L_2 was 439 μm long by 35 μm wide (Fig. 1C).

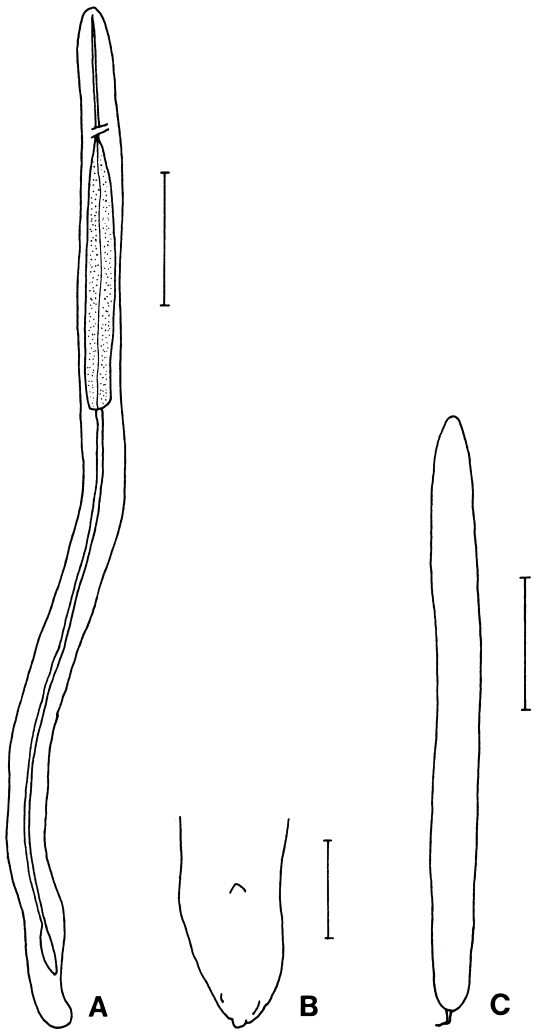

Fig. 1. Filarial larvae found in the thorax of *Simulium* species. (A) Third-stage filarial larva found in *S. nigrogilvum* (Scale bar, 100 μm). (B) Tail of the third-stage larva (same as A), ventral view (Scale bar, 25 μm). (C) Second-stage filarial larva found in *S. asakoae* (Scale bar, 100 μm).

Other nematodes found in black flies

Non-filarial nematodes were found in 1.03% (5/484) of *S. asakoae* dissected. There were at least two types of non-filarial nematodes distinguished by the characteristic shape of tail, one sharply pointed and the other bluntly ended.

DISCUSSION

In the present study, we found that in Tambol Ban Laung, Doi Inthanon National Park, Chiang Mai Province, northern Thailand, females of nine simuliid species were attracted to a human. Among them, *S. nakhonense, S. chamlongi* Takaoka et Suzuki, *S. fenestratum* Edwards, *S. chumpornense* Takaoka et Kuvangkadilok, *S. siamense* Takaoka et Suzuki and *S.* sp. E (*sensu* Takaoka et Suzuki) were collected for the first time using a human attractant.

In our previous investigation carried out in Ban Pang Fan Village (250 m in altitude), Chiang Mai Province in June (summer season), a considerable number of *S. nodosum* were attracted to feed on humans and water buffalos, and 2.3% of them were carrying filarial larvae (Takaoka *et al*., 2003). In Tambol Ban Laung (750 m in altitude), we found that *S. nigrogilvum* and *S. asakoae,* two of the three abundant species, were naturally infected with filarial larvae although their natural infection rate was very low. The low infection rate might be attributable at least partially to the cool air temperature in December (winter season) at the high altitude of the study area, which might have been too low for the development of the filarial larvae, as well as for the stimulation of blood-feeding. It might be also influenced by many factors of host animals such as population density, distribution, behavior (e.g., diurnal or nocturnal), prevalence and intensity of infection with filariae, all of which, though, remain to be studied yet.

A small number of filariae are currently known to be transmitted by black flies (Bain and Chabaud, 1986); they belong to 4 genera. Species of *Splendidofilaria* from birds can be eliminated because of the different caudal extremity and very short body of the infective larva. *Mansonella* third-stage larvae, also small, have four caudal lappets, and only *M. ozzardi* Manson from humans in South America is transmitted by simuliids (and equally by *Culicoides* spp.). *Onchocerca* species are more often transmitted by simuliids. Among the species which likely exist in cattle from Thailand, *O. lienalis* Stiles, *O. gutturosa* Neumann, *O. cebei* Galliard and *O. gibsoni* Cleland et Johnston, the first two are known to develop in simuliids. *O. lienalis* is shorter (400-560 μm long) and has a greater ratio of esophagus/body length; *O. gutturosa,* which is more frequently transmitted by *Culicoides* spp., has a higher esophagus/body length ratio and a tail differently-shaped. The caudal extremity of the infective larva with a salient axial point is found in *Onchocerca* species from African suids (Wahl and Bain, 1995), from a Japanese bovid (Takaoka and Bain, 1990; Uni *et al.,* 1998) and in the recently described infective larvae from *S. nodosum* in Thailand (Takaoka *et al.,* 2003) but the tail of these species is cylindrical and long (more than twice their width at anus); in addition this last L_3 from Thailand has a much longer body (1,315-1,500 μm long). Among *Dirofilaria* species, *D. ursi* Yamaguti does not exist in this area and its infective larva is small; *D. immitis* Leidy, most commonly transmitted by culicids but capable of developing into L_3 in *S. takahasii* Rubtsov, is similar in the lengths of whole body and esophagus to the present L_3 (Takaoka and Baba, 1987), but its tail is not conical. The present L_3, which is thick, of medium length and with a short tail, represents an unknown filaria from the local wild mammalian fauna, which might be an *Onchocerca* species, or a genus of Dirofilariinae.

The L_2 detected from *S. asakoae* also remained unidentified. It is not clear whether this L_2 is conspecific to the L_3 from *S. nigrogilvum* or not. Female *S. asakoae* has a large claw tooth, and is supposed to be ornithophilic. Therefore birds are likely to be a host of the L_2.

To summarize, this is the first report of natural infections of two black-fly species, *S. nigrogilvum* and *S. asakoae,* with a filarial larva.

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