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# A POTENT ANTIMALARIAL ACTIVITY OF HOT-WATER EXTRACTS OF PLANTS BELONGING TO THE FAMILY SAXIFRAGACEAE AGAINST *PLASMODIUM YOELII* 17XL IN ICR MICE

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**Abstract**: Hot-water extracts of 7 plants of the family Saxifragaceae; *Hydrangea macrophylla* var. *Otaksa, H. macrophylla, H. macrophylla serrata* var. *acuminata, H. involucrata, H. hirta, H. paniculata*, and *Cardiandra alternifolia*, were screened for antimalarial activity in ICR mice infected with *Plasmodium yoelii* 17XL. The leaf extract of *H. macrophylla* var. *Otaksa* or *H. macrophylla* had an antimalarial activity against rodent malaria, showing low parasitemia levels during administration. Following a transient recrudescence of malaria parasites in the bloodstream of treated mice, no parasites could be detected by a microscopic examination. Mice treated with the leaf extract of *H. macrophylla* var. *Otaksa* or *H. macrophylla* survived during the experiment, though mice in the non-treated control and other treated groups died with a gradual body weight loss from day 6 to day10 post infection. Furthermore, the antimalarial activity of the hot-water extract of *H. macrophylla* leaf extract seemed higher than that of *H. macrophylla* var. *Otaksa* leaf extract in respect of the degree of suppression of parasite multiplication and of mouse body weight loss.

**Key words**: Saxifragaceae plants, *Plasmodium yoelii* 17XL, antimalarial activity, hot-water extract

## INTRODUCTION

Malaria is still one of the major health problems in the world. The situation worsened with the emergence of chloroquine-resistant variants of *Plasmodium falciparum*, and their spread across the globe has stimulated the development of new effective treatments against malaria (Payne, 1987; Winstanley, 2000). In 1972 artemisinin was isolated from the herb *Artemisia annua* L. used in traditional Chinese medicine as remedy for chills and fever for more than 2000 years (Klayman, 1985). Isolation of artemisinin and the development of more effective derivatives from this drug (Van Agtmael *et al*., 1999) have led to further trial for identifying other medicinal compounds of potential as antimalarial agents from natural products (Iwu *et al*., 1986; Isaka *et al*., 1999; Rahman *et al*., 1999).

We thus began to investigate experimentally with rodent malaria models antimalarial activity of the plants used experientially as ingredients of various prescriptions in the traditional medical remedies in China and Japan for chills and fever as antimalarial agents. In a continuation of our research program, the hot-water extract of leaves of *Hydrangea macrophylla* var. *Otaksa* showed a high in vivo antimalarial activity against *Plasmodium yoelii* (Ishih *et al*., 2001). Furthermore the fraction including the mixture of febrifugine and isofebrifugine having antimalarial activity was isolated from leaves of *Hydrangea macrophylla* var. *Otaksa* (Takezoe *et al*., 2001). These compounds had already been isolated from *Dichroa febrifuga* (Koepfli *et al*., 1947) and from *H. umbellata* (Abolondi *et al*., 1952). From a practical viewpoint of folk medicine, it is therefore of interest to evaluate the antimalarial activity of plants of the family Saxifragaceae which are widely distributed throughout Japan and are not used as antimalarial folk medicines in Japan. This paper deals with an in vivo antimalarial evaluation of the Saxifragaceae plants using ICR mice infected with the rodent malaria parasite *P. yoelii* 17XL to seek plants with large amount of alkaloids, febrifugine and

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isofebrifugine, or with another antimalarial principles.

## MATERIALS AND METHODS

**Plant materials**

Six species were selected and tested for in vivo antimalarial activity using rodent malaria parasite. The plants specimens such as *Hydrangea macrophylla* Thunb. Ser., *H. macrophylla* Seringe subsp. *serrata* Makino var. *acuminata* Makino, *H. involucrata* Sieb., *H. hirta* Sieb. et Zucc., *H. paniculata* Sieb., and *Cardiandra alternifolia* Sieb. et Zucc., were collected in and around the campus of Hamamatsu University School of Medicine in 1999 or from the Botanical Garden, University of Shizuoka in 1999. *H.macrophylla* Seringe var. *Otaksa* Makino was also used to compare the activity with other plants.

**Hot-water extract**

*Extracts of the family Saxifragaceae plants*:

Five g of air-dried leaves or 20 g of air-dried roots of 6 plants except *C. alternifolia*, or 5 g of air-dried whole plants of *C. alternifolia* put in a herbal bag (Tokiwa Industry) was added with 500 m*l* of distilled water and boiled. After removing leaves, roots or whole plants the extract fluid was finally concentrated to 50 m*l*. Each hot-water extract (equivalent to 0.1 g/m*l* extract and 0.4 g/m*l* extract, respectively) was stored at 4℃ until use.

*Leaf extract of* H. macrophylla *or* H. macrophylla *var*. Otaksa :

Five, 2.5 and 1.25 g of air-dried leaves of each plant put in a herbal bag was added with 500 m*l* of distilled water and boiled. After removing leaves the extract fluid was finally concentrated to 50 m*l*. Each hot-water extract (equivalent to 0.1 g/m*l* extract, 0.05 g/m*l* extract and 0.025 g/m*l* extract, respectively) was stored at 4℃ until use.

**In vivo antimalarial activity of hot-water extract**

All animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University School of Medicine. Outbred male ICR mice, 8 weeks old, purchased from Japan SLC Inc., were used. For experiments, the blood stage parasites of *Plasmodium yoelii* (strain 17XL) from frozen stock were injected into two mice. The mouse showing 10-15% of parasitemia was bled under ether anaesthesia to collect *P. yoelii* 17XL-parasitized blood. Experimental mice were infected intraperitoneally with $1x10^5$parasitized erythrocytes. Infected mice treated with test samples were monitored for % parasitemia and days of survival relative to control mice up to day 60 post infection.

*Antimalarial activity of extracts of the family Saxifragaceae plants*:

To evaluate antimalarial activity of hot-water extract of plants, infected ICR mice were divided into three per group for activity assay of each hot-water extract, and five mice were used as non-treated, infected control. Starting on day 3 after injection of parasitized erythrocytes, mice were inoculated through an esophageal tube with a hot-water extract at 75 $\mu l$/10 g body weight (b.wt.) in the treated group and distilled water in the non-treated, infected one, respectively, twice a day for 5 consecutive days.

*Comparison of antimalarial activity of the leaf extract of* H. macrophylla *with that of* H. macrophylla *var*. Otaksa :

Infected mice were divided into four per group for activity assay of hot-water extract (0.1 g/m*l* extract, 0.05 g/m*l* extract or 0.025 g/m*l* extract), and five mice were used as non-treated, infected control. Starting on day 3 after injection of parasitized erythrocytes, mice were orally given a hot-water extract at 75 $\mu l$/10 g b.wt. in the treated group and distilled water in the non-treated, infected one, respectively, twice a day for 5 consecutive days.

## RESULTS

**Antimalarial activity of the hot-water extract of the family Saxifragaceae plants**

The results of the investigation of the antimalarial activity of plant extracts in vivo are summarized in Table 1. Infected mice were orally given each of 13 different extracts from 7 plants twice a day for 5 consecutive days from day 3 after inoculation of the parasitized erythrocytes. Of 13 extracts, the leaf extract of *H. macrophylla* var. *Otaksa* or *H. macrophylla* showed an antimalarial activity, although other extracts showed no effects. Mice in the non-treated control and the treated groups except one given the extract of *H. macrophylla* var. *Otaksa* or *H. macrophylla* leaves died from day 5 to day 10 post infection with a gradual body weight loss. However, in the groups given *H. macrophylla* var. *Otaksa* extract and *H. macrophylla* extract, respectively, the body weight gradually decreased from day 9 and day10, respectively, but turned to increase in several days, and all mice survived during the experiment.

**Comparison of antimalarial activity of the leaf extract of *H. macrophylla* with that of *H. macrophylla* var. *Otaksa***

As shown in Fig.1, mice in the non-treated control and groups given either 0.05 g/m*l* or 0.025 g/m*l* extracts died from day 6 to day10 post infection with a gradual body weight loss. However, in the group given 0.1 g/m*l* extract of *H. macrophylla* var. *Otaksa* leaves and *H. macrophylla* leaves, respectively, the body weight gradually decreased from day 10 and day 12, respectively, but turned to increase in a few days, and all mice survived during the experiment. Malaria parasites appeared from day 3 post infection in the bloodstream of the control and treated groups (Fig. 2). Para-

sitemia of each mouse in the control and the groups given either 0.05 g/m*l* or 0.025 g/m*l* extract of both plants gradually increased and all of the mice died by day 10. Mice treated with 0.1 g/m*l* extract of *H. macrophylla* var. *Otaksa* leaves or of *H. macrophylla* leaves showed low parasitemia levels during medication. On day 9, however, malaria parasites increased in the bloodstream of the mice treated with *H. macrophylla* var. *Otaksa* leaf extract and each peak parasitemia was more than 50%. Then the parasitemia level decreased and on day 22 parasites in the treated mice could not be detected by a microscopic examination. On the other hand, in the bloodstream of mice given *H. macrophylla* leaf extract, parasites increased on day 10, and although one mouse showed 30% of peak parasitemia, other mice showed less than 10% of parasitemia. Then the parasitemia decreased and on day 22 parasites in the treated mice could not be detected by a microscopic examination.

## DISCUSSION

Our previous study revealed the presence of compounds having an antimalarial activity in *H. macrophylla* var. *Otaksa* leaf extract (Ishih *et al*., 2001). Our present results indicate that among 7 plants belonging to the family Saxifragaceae the hot-water extract of *H. macrophylla* leaves or of *H. macrophylla* var. *Otaksa* leaves has a potent antimalarial activity against *Plasmodium yoelii* 17XL in ICR mice using approximately 5-fold dosage of clinical one prescribed in the traditional remedies in China. Hot-water extract of *H. macrophylla* leaves seems to have the same types of effects on malaria parasites in vivo as the extract of *H. macrophylla* var. *Otaksa* leaves had. Furthermore, the antimalarial activity of the hot-water extract of *H. macrophylla* leaf extract seemed higher than that of *H. macrophylla* var. *Otaksa* leaf extract in respect of the degree of suppression of parasite multiplication during medication and at a recrudescence, and of mouse body weight gain/loss. This extract exhibited marked suppression of multiplication of parasites during medication, indicating that the extract

Table Antimalarial screening of plant extracts using *Plasmodium yoelii* 17XL in ICR mice

| Plant species | Parts tested | No. mice survived /No. mice used | Ranges of survival days* |
|---|---|---|---|
| Untreated infected control | - | 0/5 | 5-8 |
| *Hydrangea macrophylla* | Leaves | 3/3 | 60 |
| Seringe var. *Otaksa* Makino | Roots | 0/3 | 5-7 |
| *Hydrangea macrophylla* | Leaves | 3/3 | 60 |
| Thunb. Ser. | Roots | 0/3 | 4-5 |
| *H. macrophylla* | Leaves | 0/3 | 5 |
| Seringe subsp. *serrata* Makino | Roots | 0/3 | 4-6 |
| var. *acuminata* Makino | | | |
| *H. involucrata* Sieb. | Leaves | 0/3 | 5-6 |
| | Roots | 0/3 | 5 |
| *H. hirta* Sieb. et Zucc. | Leaves | 0/3 | 5-7 |
| | Roots | 0/3 | 5-9 |
| *H. paniculata* Sieb. | Leaves | 0/3 | 5-6 |
| | Roots | 0/3 | 5 |
| *Cardiandra alternifolia* | Whole plants | 0/3 | 5-6 |
| Sieb. et Zucc. | | | |

Mice were orally given a hot-water extract at 75 $\mu l$ /10 g body weight in the treated group and distilled water in the untreated, infected one, respectively, twice a day for 5 consecutive days from day 3 post infection with $10^5$ *P. yoelii* 17XL-parasitized erythrocytes.
*Number of surviving mice was monitored until day 60 post infection.

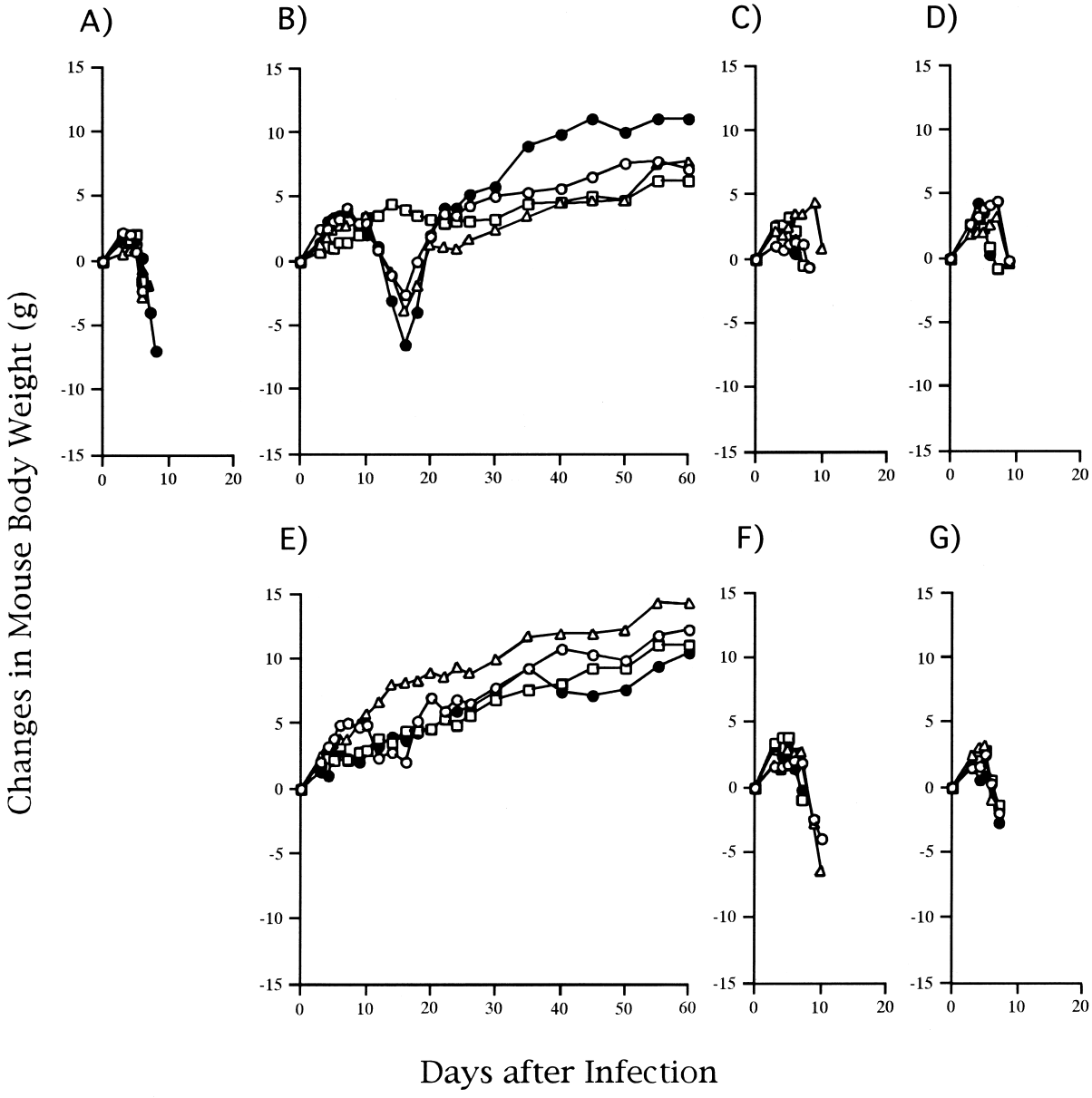


Figure 1 Body weight profile of each mouse in response to treatment with 3 different concentrations of hot-water extracts (at 75 $\mu l$ /10 g b.wt.) of *H. macrophylla* var. *Otaksa* and *H. macrophylla*. *P.yoelii* 17XL-infected mice were orally given leaf-extract, twice a day, for 5 consecutive days from day 3 to day 7 after inoculation intraperitoneally with $10^5$ parasitized erythrocytes. Control group (A) of mice received an equivalent volume of water. Each symbol represents an individual mouse. *H. macrophylla* var. *Otaksa* leaf extract; 0.1 g/m*l* (B), 0.05 g/m*l* (C), 0.025 g/m*l* (D): *H. macrophylla* leaf extract; 0.1 g/m*l* (E), 0.05 g/m*l* (F), 0.025 g/m*l* (G).

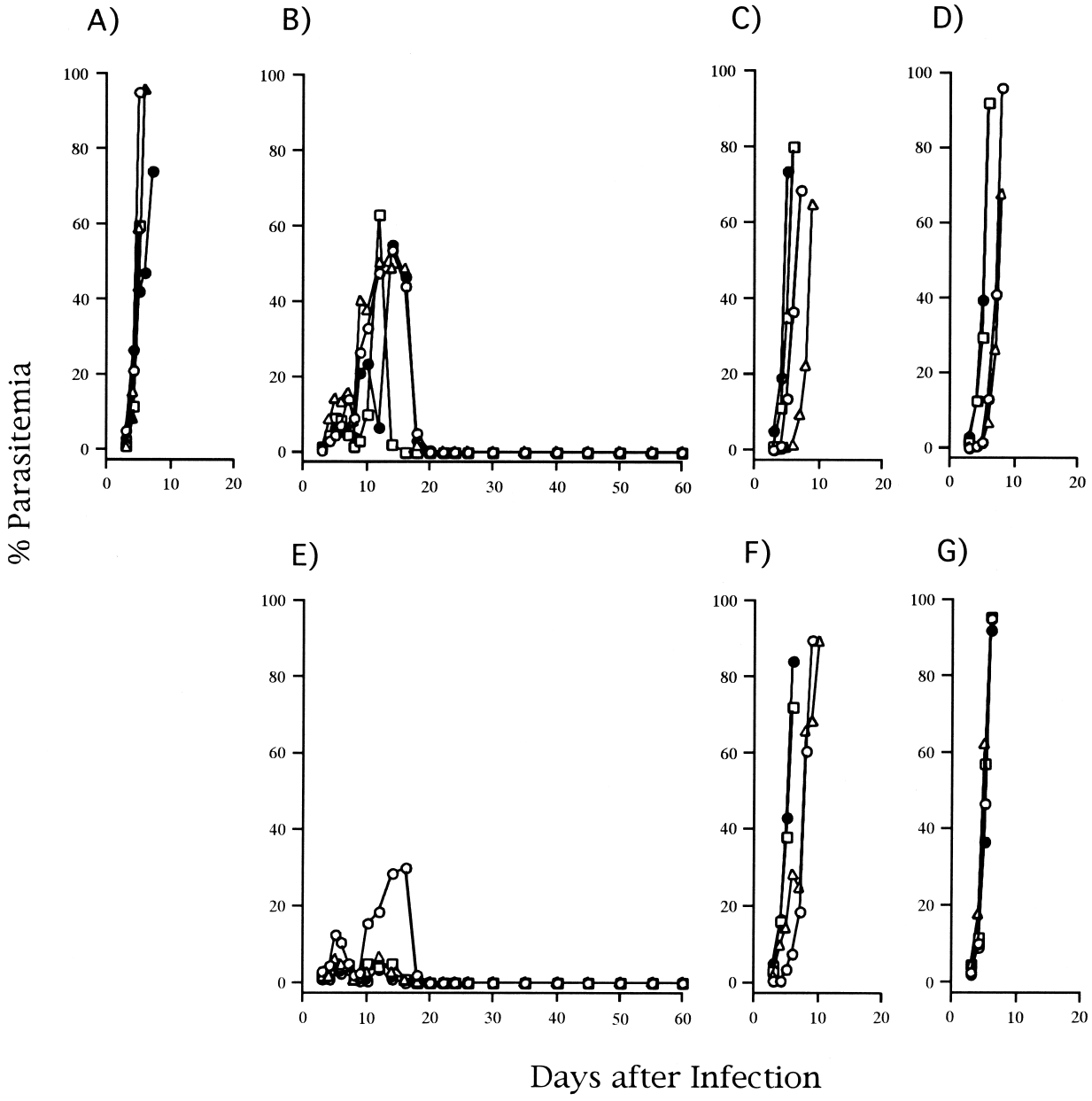


Figure 2 Parasitemia profile of each mouse in response to treatment with 3 different concentrations of hot-water extracts (at 75 $\mu l$ /10 g b.wt.) of *H. macrophylla* var. *Otaksa* and *H. macrophylla*. *P.yoelii* 17XL-infected mice were orally given leaf-extract, twice a day, for 5 consecutive days from day 3 to day 7 after inoculation intraperitoneally with $10^5$ parasitized erythrocytes. Control group (A) of mice received an equivalent volume of water. Each symbol represents an individual mouse. *H. macrophylla* var. *Otaksa* leaf extract; 0.1 g/m*l* (B), 0.05 g/m*l* (C), 0.025 g/m*l* (D): *H. macrophylla* leaf extract; 0.1 g/m*l* (E), 0.05 g/m*l* (F), 0.025 g/m*l* (G).

has a direct action on parasites. Most of the antimalarials work against the late trophozoite and schizont stage of the malaria parasite and thus the number of asexual parasites decreases. Artemisinin derivatives also work against early trophozoite and ring stages (Van Agtmael *et al*., 1999). Febrifugine and isofebrifugine being active principles against malaria were isolated from the roots of *D. febrifuga* used in Chinese traditional medicine as a treatment for fever and malaria (Kuehl *et al*., 1948; Takaya *et al*., 1999), and *H. macrophylla* var. *Otaksa* and *H. umbellata* belong to the family Saxifragaceae including *D. febrifuga*. Thus it is possible that febrifugine and isofebrifugine are the common active alkaloids consisting in the Saxifragaceae palnts and the content of principles varies in each plant species. Furthermore, the root extract of *H. macrophylla* var. *Otaksa* and *H. macrophylla* showed no antimalarial activity, and hence it is of interest to investigate the distribution of effective alkaloids within plants. Since the mechanisms of action of hydrangea compounds are still unknown, the investigation of the morphologic changes induced in malaria parasites by the mixture of febrifugine and isofebrifugine will be started.

In the mice treated with the hot-water extract of *H. macrophylla* var. *Otaksa* or *H. macrophylla* leaves, parasitemia in the bloodstream decreased after a transient recrudescence and malaria parasites could not be detected by microscopic observation, suggesting that the elimination of malaria parasites after a recrudescence might be associated with host immune mechanisms. It is now generally accepted that cell-mediated immune mechanisms contribute to acquired immunity to blood-stage malaria. Studies are in progress to investigate the induction of T helper cell subsets during the course of resolution of primary infection in mice treated with the mixture of febrifugine and isofebrifugine.

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# FIELD EVALUATION OF A RAPID IMMUNOCHROMATOGRAPHIC TEST 'PARACHEK-F' IN A POST-MONSOON *PLASMODIUM FALCIPARUM* MALARIA OUTBREAK IN VILLAGES OF SOUTH INDIA

## Indigenous diagnostic test kit for *P. falciparum*

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**Abstract**: An indigenous rapid immunochromatographic test Parachek-F for diagnosis of *Plasmodium falciparum* malaria was evaluated by the field staff in a post-monsoon *P. falciparum* malaria outbreak in villages of district Raichur, Karnataka, South India in November 1999. The test functions based on dipstick *P. falciparum* histidine rich protein-2 (PfHRP-2) antigen capture assay. Of the 232 uncomplicated clinically diagnosed malaria cases, 158 (68.1%) were positive for malaria by microscopy of JSB-stained thick blood smears. Of these, 13 were infected with *P. vivax*, 140 with *P. falciparum* and 5 had mixed infections of *P. vivax* and *P. falciparum*. Malaria patients were treated with age-specific oral doses of quinine followed by primaquine. Taking microscopy as gold standard, Parachek-F detected PfHRP-2 antigen in 136 samples (ratio 0.93) and was 93.1% sensitive and 98.8% specific. Positive predictive value, negative predictive value and efficacy were 99.2%, 89.6% and 95.2% respectively. No cross reactivity was observed with *P. vivax* infection. False negative interpretation was associated in 40% (10/25) lower-grade parasitaemias (parasitaemia <100/$\mu l$ blood) where sensitivity was only 60%. False positive result was associated in 1 case (1/74). Cases showing false negative results had taken presumptive treatment with chloroquine prior to the test. Careful microscopical examination on thin smears of such cases demonstrated that the morphology of the parasites was abnormal and distorted indicating the parasites were affected by chloroquine. The possible role of chloroquine resulting false negative results is suggested in this communication. Positive correlation between test bands intensity and parasite density was observed ($r$=0.137; P<0.05). The test is indigenously developed, rapid, simple in its application and was found suitable for field condition. Parameters like patients' conditions, history of drug intake, morphology of parasites at different developmental stages are to be considered for evaluation of such tests.

**Key words**: Presumptive Treatment, PfHRP-2, Immunochromatographic test, Sensitivity, Specificity

## INTRODUCTION

In most parts of India malaria is unstable and has re-emerged in epidemic forms with high mortality. In the last decade an increase of about 40% in *Plasmodium falciparum* infection has been recorded. Almost all the deaths are caused by *P. falciparum* infection and delayed treatment (Sharma, 1999). Malaria case detection and treatment is performed by the National Anti Malaria Programme (NAMP) through active and passive surveillance under primary health care system. A single dose of presumptive treatment (600 mg chloroquine; adult dose) is administered to all fever cases at the time of taking of blood samples followed by radical treatment to the malaria positive cases. The diagnosis is based on microscopical examination of JSB-stained (Singh and Bhattacharya, 1944) whole blood by skilled personnel. In all best circumstances, it lags some important time and thus delays in the delivery of radical

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treatment. The early diagnosis of *P. falciparum* malaria and institution of prompt treatment was emphasized for reducing morbidity and mortality (WHO, 1993). In such situation, test system developed by the non-microscopic antigen detection of *P. falciparum* histidine-rich protein-2 (PfHRP-2) by means of an immunological capture assay has proven to be very encouraging. The test is rapid, simple and a small amount of whole blood is required. It does not require any instrument or electricity, thus found suitable for rural setup also. The result is very specific, sensitive and can be read with the naked eyes. Evaluation of several commercially available test kits e.g., *Para* Sight™ F test (Karbwang *et al*., 1996; Singh *et al*., 1997a) and ICT Malaria® Pf test (Kumar *et al*., 1996; Singh *et al*., 1997b; Valecha *et al*., 1998; Ghosh *et al*., 2000) and Determine™ (Singh and Valecha, 2000a) have confirmed the usefulness of these rapid tests. Recently, an indigenous rapid test kit Parachek-F has been developed (Kumar *et al*., 2000). This test kit, like other systems functions similar to the antigen capture assay which detects the circulating histidine-rich protein-2 (HRP-2) antigen released by *P. falciparum* (Garcia *et al*., 1996). An initiative was taken by NAMP to introduce the rapid diagnostic test in the national programme. In the present study, this kit was evaluated by field staff with minimal orientation under field condition, considering microscopy as gold standard, in a post-monsoon outbreak of malaria mostly of *P. falciparum* in villages of district Raichur, Karnataka, South India.

## MATERIALS AND METHODS

*Study area*

The Parachek-F test was conducted in two villages of two Primary Health Centres (PHCs) of district Raichur, south Karnataka during an outbreak of malaria mainly due to *P. falciparum* in November 1999. Village Ramdurg (population 2,528) of PHC Gabbur and village Masarkal (population 1,476) of PHC Masarkal were selected for the study. Generally villages in this area are large, distantly located, semi-town type, having population around 1,500 to 2,500 and well connected by good roads. Each village is surrounded by small rocky hillocks and vast irrigated land. Open dug wells and rain fed irrigation tanks are the main source of water. Escape channels from the tanks and seepage water from the hillocks form run-off stream along the village side. Malaria vectors *Anopheles culicifacies, An. fluviatilis* and occasionally *An. stephensi* were found to breed in such breeding habitats. The area is dry-deciduous with prevailing hot climate. Temperature ranges between 13℃ and 44℃. Annual rainfall ranges from 800 to 1,200 mm and precipitates mainly in the post-monsoon period. Irrigation is restricted during post-monsoon period. Millets and cotton are the main cash crops. Rice is cultivated in the catchment area.

Intensive malaria control activities with fogging of malathion (5% technical) and indoor residual spraying with deltamethrin (2.5% wp) and presumptive treatment with chloroquine to all fever and fever related cases were initiated at the time of investigation. All age and sex groups including pregnant women having recent febrile illness were enrolled in the study. The test was performed also on those cases who had received presumptive treatment with chloroquine two to three days before. Such cases wanted immediate results since the reports of blood smear examination were awaited. Both *P. vivax* and *P. falciparum* species were present and both chloroquine and sulfadoxine-pyrimethamine were found resistant in *P. falciparum* (Ghosh and Sathyanarayan, 2000).

*Work plan*

The test was conducted under field conditions by the field staff of Malaria Research Centre, Bangalore. Two scientists, one microscopist and two field health workers were involved in the test evaluation. One scientist was assigned to enroll names, history of fever, nature of ailments, any intake of anti-malarial drugs from each patient. One worker was engaged on drawing two sets of blood smears and the other performed the Parachek-F test. The microscopist stained the blood smears and examined on the spot. At the end, the second scientist correlated the thick smear results and Parachek-F test. In this way, the test procedure was blinded at each level. The final readings were rechecked by the first scientist. Local health staff and village leaders (*Gram Pradhans*) assisted in carrying out the tests. Verbal consent of each patient was obtained.

*Microscopy of JSB-stained blood smears*

Two sets of blood smears (both thick and thin) were prepared and stained with JSB stain. JSB stain is a combination of two aqueous solutions. The first solution (JSB I) contains microscopic methylene blue and the second one aqua yellowish eosin (JSB II). It is a rapid stain. First the thin smears were fixed with methanol and the thick were dehaemoglobinised by putting simply in distilled water. After completely drying both the smears were dipped into JSB II solution for 2-3 seconds and subsequently washed thoroughly in buffer water (phosphate buffer; pH 6.8). The slides were then dipped in JSB I solution for 45 seconds. It was again washed gently and thoroughly in the buffer. The stained slides were allowed to dry. One set was examined on the spot under a compound microscope (Zeiss KF-2, Germany) at 1,000 x magnification. The second set was

stained in the laboratory at Bangalore and kept for reference. A smear was declared negative after counting a minimum of 200 micro fields on thick smears. Thin smears were examined for final identification of the species of parasite. All the blood smears including discordant results were thoroughly re-examined by the senior author (SKG). Parasites from each positive case were counted against 300-500 white blood cells (WBCs). The parasitaemia was calculated assuming 8,000 WBCs/$\mu l$ blood. *P. falciparum* cases were treated with age-specific oral doses of quinine sulphate (adult dose : 600 mg 8 hourly for 7 days) followed by a single dose of 45 mg primaquine. *P. vivax* cases were treated with chloroquine (1,500 mg over 3 days) following by 75 mg primaquine in 5 days. Primaquine was not given to the infants and pregnant women.

*Malaria diagnosis by Parachek-F*

The commercial diagnostic kit Parachek-F, indigenously developed by Orchid Biomedical Systems, Verna, Goa, India was used for rapid diagnosis of *P. falciparum* malaria. The kits were supplied to the State Health Department by NAMP for field evaluation. The test system uses two mice antibodies specific against the immunodominant repeating sequence from HRP-2 molecules. One antibody is conjugated to visible colloidal gold and impregnated into the sample pad while the second one is immobilized in a line on the nitrocellulose strip. The test was performed following strict manufacturer's instruction (Fig. 1). The kits sealed in pouches were stored at 4℃ in the PHC and brought to room temperature before carrying out the tests. One drop of finger pricked whole blood (~5$\mu l$) was applied directly on the sample pad just below the arrow marks on the test strip. The strip was dipped in 4 drops of clearing buffer in a clean 12 x 75 mm test tube keeping the arrows pointing downwards. In the positive samples pink coloured bands were formed along with control bands within 4-5 minutes. In the negative samples only control bands were noticed. Presence of control bands validated the tests performed. The final readings were taken after 15 minutes. The positive test bands were graded (+1 to +4) based on the colour intensity.

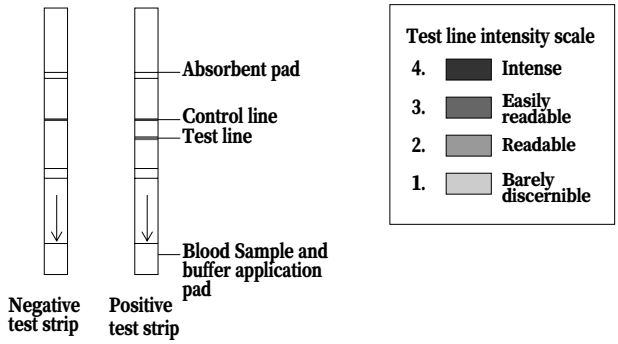


Fig. 1: Parachek-F test strip.

*Data analysis*

To evaluate Parachek-F test, the result of thick smear examination was taken as gold standard. Variables like number of true positives (positive reactors in the assay with positive microscopy; TP), number of true negatives (negative reactors in the assay with negative microscopy; TN), number of false positives (positive reactors with negative microscopy; FP) and number of false negatives (negative reactors with positive microscopy; FN) were measured for calculating sensitivity, specificity, positive and predictive values and efficacy of the test (Tjitra *et al*., 1999). Sensitivity was thus calculated as TP/(TP + FN), specificity as TN/(TN + FP), positive predictive value (PPV) as TP/(TP + FP) and negative predictive value (NPV) as TN/(FN + TN). Test efficacy was defined as (TP + TN)/ total number of cases.

For the purpose of analysis, mixed infection cases having *P. vivax* and *P. falciparum* were treated as *P. falciparum*. As the subjects having *P. falciparum* gametocytes only are considered positive cases in the national programme and has epidemiological implications, these cases were considered as true positives. All *P. vivax* cases were treated as negative.

The correlation between test band intensity and the parasite density was analyzed using Microstat software (Ecosoft. Inc. USA) and significance was determined using F test considering $P < 0.05$ to be significant.

## RESULTS

Of the total 232 subjects enrolled in the study (133 from Ramdurg and 99 from Masarkal), 128 were males and 104 females. Prevalence of malaria with age wise breakdown given in Table 1 revealed that transmission of malaria was active at the study area. The youngest one was three months female and the eldest one was 80 years male. Microscopical examination on thick smears demonstrated 158 (68.1%) were positive for malaria parasites. Of these, 13 were infected with *P. vivax*, 140 with *P. falciparum* and 5 had mixed infections of *P. vivax* and *P. falciparum*. Of the 145 *P. falciparum* cases, 107 were with rings only, 32 with rings and gametocytes and 6 with gametocytes only.

The Parachek-F test detected PfHRP-2 antigen in 136 (ratio 0.93) *P. falciparum* patients and was non-reactive to *P. vivax* cases (Table 2). Considering *P. falciparum* rings only, the test detected 99 of 107 cases (ratio 0.92); while in the group with gametocytes only 4 out of 6 cases were detected (ratio 0.66). All the 32 cases having concomitant

Table 1 Prevalence of malaria in the affected villages of district Raichur, south Karnataka in November 1999

| Patients age Groups (years) | Blood smear examined | Positive for malaria | *P. vivax* | *P. falciparum* | *P. vivax*+ *P. falciparum* | Slide Positivity Rate | Slide falciparum Rate | *P. falciparum* Percentage |
|---|---|---|---|---|---|---|---|---|
| ≤1 | 7 | 3 | 2 | 1 | 0 | 42.85 | 14.28 | 33.33 |
| > 1 - ≤ 4 | 17 | 12 | 1 | 10 | 1 | 70.58 | 64.70 | 91.66 |
| > 4 - ≤ 8 | 24 | 21 | 1 | 19 | 1 | 87.50 | 83.33 | 95.23 |
| > 8 - ≤ 14 | 31 | 21 | 3 | 17 | 1 | 67.74 | 58.06 | 85.71 |
| > 14 | 147 | 98 | 5 | 91 | 2 | 66.66 | 63.26 | 94.89 |
| Pregnant Women | 6 | 3 | 1 | 2 | 0 | 50.00 | 33.33 | 66.66 |
| Total | 232 | 158 | 13 | 140 | 5 | 68.10 | 62.50 | 91.77 |

Table 2 Performance of Parachek-F assay in 232 clinically diagnosed malaria patients

| | No. Plasmodia positive by | | |
|---|---|---|---|
| | Thick smear | Parachek-F | Ratio |
| *P. falciparum* | 140 | | |
| *P. vivax* | 13 | 0 | |
| *P. vivax*+*P. falciparum* | 5 | 5 | |
| Total positive | 158 | 136 | 0.86 |
| Total negative | 74 | 96 | |
| *P. falciparum* only | | | |
| Rings only | 107 | 99 | 0.92 |
| Rings + Gametocytes | 32 | 32 | 1.00 |
| Gametocytes only | 6 | 4 | 0.66 |
| Total positive taking all *P. falciparum* cases | 145 | 136 | 0.93 |

Table 3 Efficacy of Parachek-F test

| | | Microscopic diagnosis | |
|---|---|---|---|
| | | + | - |
| Parachek-F diagnosis | + | 135 (TP) | 1 (FP) |
| | - | 10 (FN) | 86 (TN) |

Sensitivity = 135/(135+10) = 0.931; Specificity = 86/(86+1) = 0.988; Positive predictive value = 135/(135+1) = 0.992; Negative predictive value = 86/(86+10) = 0.896; Efficacy = (135+86)/(135+10+1+86) = 0.952.
All *P. falciparum* gametocyte cases considered as positive *P. vivax* cases considered as negative.
TP - True positive, FP - False positive,
FN - False negative, TN - True negative

Table 4 Sensitivity of Parachek-F test by parasitaemia

| Parasitaemia/ $\mu l$ blood | Thick smear diagnosis | Parachek-F diagnosis | Sensitivity (%) |
|---|---|---|---|
| 17 - 100 | 25 | 15 | 60 |
| 101 - 500 | 14 | 14 | 100 |
| 501 - 1,000 | 16 | 16 | 100 |
| 1,001 - 2,000 | 53 | 53 | 100 |
| 2,001 - 5,000 | 23 | 23 | 100 |
| > 5,000 | 12 | 12 | 100 |
| All *P. falciparum* cases | 145 | 146 | 99.2 |

rings and gametocytes showed positive to both microscopy and PfHRP-2 antigen (ratio 1.00). Thus the sensitivity of PfHRP-2 assay was 93.1%, the specificity 98.8%, the positive predictive value 99.2%, the negative predictive value 89.6% and the test efficacy was 95.2% respectively. Prior to the test, 19/135 TP, 10/10 FN, 1/1 FP and 12/86 TN (total 42/232) cases consumed chloroquine as presumptive treatment (Table 3).

Details of parasitaemia in the *P. falciparum* cases and related sensitivity are summarized in Table 4. Parasitaemia ranged from 17 to 57,120 parasites/$\mu l$ blood. Compared to microscopy, Parachek-F failed to detect infection in 10 of 25 patients (40%) with low parasitaemia (<100 parasites/$\mu l$ blood) where 8 were with rings only and 2 with gametocytes only. Thus, the test demonstrated 60% sensitive in lower-grade parasitaemias and 100% in cases having>100 parasites/$\mu l$ blood. Careful microscopical examination on thin smears in the lower-grade parasitaemias revealed gross distortion of the parasite morphology. The rings were very small, nugget shaped, chromatin and cytoplasm were compact due to lack of vacuole. The morphology of the gametocytes was also altered having oval and slightly elongated shapes. The pigments and chromatin were dispersed, distributed in the cytoplasm.

Details of correlation between test band intensity and stage wise parasite density is shown in Table 5. Positive correlation ($r$=+0.137; $P$ <0.05) was noticed in all stages of parasite except in cases having only gametocytes ($r$=-0.749; $P$ >0.05) and band intensity grade 2 ($r$=-0.306; $P$ >0.05).

## DISCUSSION

In the study area malaria was not a serious problem in the recent past (NAMP, 1998). In the beginning of the study, there was a confusion of the cause of outbreak even though

Table 5 Correlation between parasite density of *P. falciparum* and colour intensity of test bands along with different stages of parasite in the test samples.

| Parasite Density Mean ± SD (Range) | Band Intensity | Stage of Parasite | *r* value | F ratio | *P* value |
|---|---|---|---|---|---|
| Band wise | | | | | |
| 56.60±25.27 (17-106) | 0 | R (n=8), G (n=2) | + 0.055 | 50.15 | 0.0001 |
| 843.53±1,821.66 (0-9,420) | 1 | Neg. (n=1), R (n=18) G (n=2), RG (n=5) | + 0.330 | 5.56 | 0.0223 |
| 2,379.67±7,384.31 (55-41,320) | 2 | R (n=25), G (n=1), RG (n=4) | - 0.306 | 3.11 | 0.0831 |
| 2,180.59±4,691.93 (54-27,290) | 3 | R (n=21), RG (n=11) | + 0.296 | 6.89 | 0.0109 |
| 6,686.79±2,133.01 (220-57,120) | 4 | R (n=35), G (n=1), RG (n=12) | + 0.240 | 14.56 | 0.002 |
| Stage wise | | | | | |
| 605.0±1,293.54 (45-3,245) | (0-4) | G only (n=6) | - 0.749 | 1.30 | 0.279 |
| 3,776.11±9,388.23 (17-57,120) | (0-4) | R only (n=107) | + 0.812 | 17.28 | 0.0004 |
| 2,461.90±4,693.75 (104-27,290) | (1-4) | RG (n=32) | + 0.159 | 8.78 | 0.004 |
| 33,254±8,384.81 (17-57,120) | (0-4) | All *P. falciparum* cases | + 0.137 | 23.17 | 0.0002 |

R - rings, G - gametocytes, Neg. - Negative for malaria.

all anti-malarial measures had initiated. The Parachek-F test clearly identified that the outbreak was due to *P. falciparum* malaria showing high sensitivity of 93.1% which is in agreement with similar commercial kits like *Para* Sight™-F (Beadle *et al*., 1994; Karbwang *et al*., 1996; Singh *et al*., 1997a) and ICT-Pf (Kumar *et al*., 1996; Singh *et al*., 1997 b; Valecha *et al*., 1998; Ghosh *et al*., 2000). Other values like specificity, positive and negative predictive values and efficacy of the test were comparable. Kumar *et al*. (2000) also demonstrated similar results with the same Parachek-F kit.

As compared to Parachek-F, *Para* Sight™-F and ICT-Pf have several washing processes. In this regard, Parachek-F is very simple and easy to operate in the field by the health workers (Fig. 1). Due to its simplicity, it is cost-effective also. Each kit costs approximately U.S.$ 0.64 which is cheaper than other commercial kits viz. *Para* Sight™-F and ICT-Pf (between U.S.$ 2.91 and 3.33) but higher than the suggested price of U.S.$ 0.40 by H. Engers (Singh and Valecha, 2000a). To make accessible, efforts should be made to produce such kits locally specially in the developing countries where malaria (mainly *P. falciparum*) is a real problem. In India, cold chain system has been provided in each PHC under primary health care. Thus, there will not be any problem to introduce this test system in the national programme.

There are some limitations in this test for poor interpretation in lower-grade parasitaemias. Low parasitaemia are most common in many areas where anti-malarial drugs are easily available and people take self-medication. Such situation is very common among travellers who are advised to cover with chemoprophylaxis (WHO, 1996). Funk *et al*. (1999) reported false negative results among travellers up to 72.0% in ICT-Pf and 29.6% cases in *Para* Sight F where parasitaemia level was < 0.1%. They did not find these kits suitable for self-diagnosis. Beadle *et al*. (1994) observed in a field test in Kenya that sensitivity and specificity markedly decreased in lower-grade parasitaemias. We have observed false negative results in 40% cases having parasitaemia level <100 parasites/$\mu l$ blood. Karbwang *et al*. (1996) observed a lack of correlation between the amount of PfHRP-2 antigen and small ring form parasitaemia. Better correlation was observed with the trophozoite load (late rings). Beadle *et al*. (1994) reported that PfHRP-2 antigen is produced in trophozoites and early gametocytes. Recently, PfHRP-2 has been reported to mediate haemozoin formation, which could be inhibited by chloroquine (Sullivan *et al*., 1996). Pandey *et al*. (1997) have demonstrated that

hexapeptide repeats Ala-His-His-Ala-Ala-Asp in PfHRP-2 are heme binding sites in this protein. It could be possible that in the early developmental stages of the parasites, PfHRP-2 molecules are not expressed due to the effect of chloroquine and demonstrate false negative results. The patients who showed false negative results showed clinical symptoms for malaria and had consumed presumptive treatment with chloroquine prior to the test. This was confirmed by the health workers who administered the presumptive treatment. Further, the morphology of the parasites in these cases clearly indicated that they were affected by chloroquine as described by Macomber and Sprinz (1967). Karbwang *et al*. (1996) observed very low specificity of 50% in *Para* Sight™ F test for detection of multi-drug resistant *P. falciparum* treatment failure as against 60% in our study cases underwent presumptive treatment. This is possibly due to the drug affected parasitaemia phenomenon.

One false positive case (1/74) detected by Parachek-F was treated 11 days before the test. This is possible when persistence of antigenemia in the patient persists even after the curative treatment (Singh *et al*., 1997a, b; Valecha *et al*., 1998; Kumar *et al*., 2000). Recently, Grobusch *et al*. (1999) has reported false-positive results in the presence of rheumatoid factors. False-positive test results could lead to inappropriate treatment of other important causes of fever. However, this should not be taken as limitation to the test because timely treatment in many sub-microscopic level parasitaemias in known malarious areas may prove fruitful.

There are mixed reports on the correlation between the test band intensity and parasite density. Some reported positive correlation (Kumar *et al*., 1996; Ghosh *et al*., 2000), while others negative correlation (Singh *et al*., 1997a, Valecha *et al*., 1998). Kumar *et al*. (2000) reported negative correlation with the same kit as against positive correlation in our study.

Recently, test kits for diagnosis of both *P. falciparum* and *P. vivax* are available commercially. One such kit (ICT P.f./P.v.) was evaluated by Singh *et al*. (2000b) in Madhya Pradesh, Central India. The test was 97.5% sensitive and 88% specific for *P. falciparum*. For *P. vivax* the sensitivity was only 72% and 99% specific. The authors concerned about the decline in sensitivity <300 parasites/$\mu l$ for *P. falciparum*. The test failed to diagnose *P. vivax* infection when the parasitaemia was $\leq$ 1500 parasites/$\mu l$. Tjitra *et al*. (1999) also made similar observation.

In conclusion, rapid immunodiagnostic tests are very useful in the field and in emergencies even having some limitations, because all test systems do have some self-limiting factors. Proper caution has to be taken for those subjects undertaking chemoprophylaxis (especially pregnant women and travellers). The test systems have additional advantage as the results can be kept as reference for a fairly long period of time. All the test cards of our study are easily legible even after more than one and half years of testing. For evaluation of such test systems, it is very important to consider parameters like patients' condition, history like drug intake, parasite morphology, etc. However, this cannot replace the microscopical diagnosis for routine epidemiological studies where different stages of parasites, their density etc. are important components, which cannot be performed by such test systems.

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# AN ANTIGENIC PEPTIDE OF MYOSIN HEAVY CHAIN-LIKE PROTEIN FROM *TRICHINELLA SPIRALIS*

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**Abstract**: We produced antigenic peptide of *Trichinella spiralis* newborn larvae (NBL) which seemed to be a part (approximately one fourth) of myosin heavy chain, and some basic profiles were revealed. The cDNA library was constructed from NBL and immunoscreened with an antibody against the parasite. A clone, designated NBL21, was selected. It contained a cDNA transcript of 1656 bp in length, which encoded 552-amino acids (64868 Da in the estimated molecular weight). The fusion protein encoded by the clone NBL21 was produced in an *Escherichia coli* expression system and affinity purified. NBL21 fusion proteins migrated at 64 kDa and reacted to *T. spiralis*-infected mouse sera and the antibody against NBL crude antigen. Antisera were developed against NBL21 fusion proteins, which reacted to a single band migrating at 200 kDa on Western blotting analysis of crude extracts from muscle larvae, and reacted to hypodermal muscles of *T. spiralis* on immunohistochemical staining. The antigen was recognized by the mouse serum obtained from the early phase of infection, but the antigenicity was devoid of species specificity.
**Key words**: Myosin heavy chain, *Trichinella spiralis*, Antigen, Fusion protein

## INTRODUCTION

*Trichinella spiralis* is a parasitic nematode that infects the skeletal muscle cells of mammals. Many questions remain to be answered about this parasite. Examples include how they evade host immune attack, and how they can establish parasitism in the host muscle cell. Moreover, the vaccine against this parasite is still far from routine usage.

For progress towards these goals, fusion proteins of *Trichinella* have been produced with some success. Vayssier *et al*. (1999) produced a protein from *T. britovi*, named as Tb Hsp70, which is a kind of heat shock protein with a molecular weight of 70 kDa. Sun *et al*. (1994) produced a protein Ts39 FP which is a potentially valuable antigen for vaccine development. Lindh *et al*. (1998) produced fusion protein which has properties of a myogenic repressor, and Nagano *et al*. (2001a) produced fusion protein with serine protease inhibitor activity.

In this study we produced a part of myosin heavy chain-like protein, and revealed some basic profiles including amino acid sequence and antigenicity. This is the first report on antigenic peptide of *Trichinella* myosin heavy chain which may afford better understanding of this conservative protein in *Trichinella* related research.

## MATERIALS AND METHODS

### *Parasites*

*T. spiralis* (ISS 413) and *Trichinella pseudospiralis* (ISS 13) were maintained in mice through oral infection. Muscle larvae were obtained by a conventional digestion method (Despomier, 1975). Newborn larvae (NBL) of *T. spiralis* were obtained through incubation of five day old adults in minimum essential medium (MEM) + 10% fetal calf serum (FCS) + antibiotics (100 $\mu l$ of streptomycin and 100 unit of penicillin per 100 m$l$ of MEM). The NBL were frozen and stored until use.

### *Crude extracts and ES products*

Larvae of *Trichinella* at the muscle stage were isolated from mice at 2 months post infection using digestion method. Crude saline extracts of larvae and ES products were prepared by conventional methods (Wakelin *et al*., 1994; Wu *et al*., 1998).

### *Preparation of antisera*

Polyclonal antibodies against crude extracts of the NBL were collected from BALB/c mice injected subcutaneously with 30 $\mu$g of the crude extracts and Freund's complete adjuvant followed by two booster injections of 15 $\mu$g of the crude extracts mixed with Freund's incomplete adju-

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vant at 2-week intervals.

Infected sera of the parasites and polyclonal antibodies against ES products were prepared by conventional methods (Nagano *et al*., 2001a, b).

*cDNA library construction*

Construction of a cDNA library from *T. spiralis* NBL was performed according to our previous method (Nagano *et al*., 1999). In brief, frozen NBL were homogenized in an extraction buffer (Quickprep micro mRNA purification kit, Amersham Pharmacia Biotech, Tokyo Japan), and mRNA was isolated by affinity chromatography using oligo (dT) cellulose.

Synthesis of cDNA from the resulting mRNA was performed using a Timesaver cDNA synthesis kit (Amersham Pharmacia Biotech) as described by the manufacturer's instructions. Resulting cDNAs were ligated into the λZAP II vector (Stratagene, La Jolla, California USA). The ligates were packaged in Gigapack Gold III packaging extract (Stratagene).

*Immunoscreening*

*E. coli* XL1-Blue MRF' strain infected with the λZAP II vector was cultured on the NZY agar plates for 4 hr at 42 ℃. And nitrocellulose membrane infiltrated with isopropyl-β-D-thiogalactopyranoside (IPTG) was overlaid for 4 hr at 37 ℃. After blocking with 5% skim milk in phosphate buffered saline (PBS) overnight at 4 ℃, the membrane was reacted with polyclonal antibodies (1/100 diluted) against *T. spiralis* NBL. Goat anti-mouse IgG alkaline phosphatase conjugate was then used as the second antibody, and the membrane was incubated in 5-bromo-4-chloro-3-indolyl-p-toluidine salt (BCIP) and nitroblue tetrazolium (NBT) for development. The positive 66 clones were picked up and re-screened three times.

*DNA sequence and homology search*

The clone NBL21 was subjected to sequencing in which the Thermo Sequenase Cycle Sequencing Kit (Amersham) with bi-directional sequencing protocol and IRD-800 and IRD-700 labeled primers were used. The products were sequenced using an automatic sequencer (Model LIC-4200 L, Aloka, Tokyo Japan). The DNA sequences were assembled and analysed by using DNASIS software (Hitachi Software Engineering, Tokyo Japan). The NCBI BLAST network service at the National Center for Biotechnology Information was used to search for the sequence homology.

*Production of fusion protein*

The NBL21 clone was subcloned into the expression vector (pTrc His, Invitrogen, California USA) and transfected to *E. coli* (DH5α, Toyobo CO. LTD., Tokyo Japan) according to the manufacture's instructions. Transformants were grown in bacterial culture medium at 37 ℃. To facilitate protein production, IPTG was added to give a final concentration of 1 mM, and cells were incubated for 3 hr at 37 ℃. The induced cells were fractured by ultrasound in 20 mM Tris HCl buffer (pH 8.0). The resultants were precipitated and the pellet was lysed by lysis buffer (20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine hydrochloride). The fusion protein was affinity purified using a HisTrap kit (Amersham Pharmacia Biotech) as described by the manufacturer.

*Production of antibodies against the fusion protein*

To produce antibodies against the fusion protein NBL 21, BALB/c mice were injected subcutaneously with 30μg of the fusion protein and Freund's complete adjuvant. In order to boost the antibodies, after two weeks, the mice were injected with 15μg of the protein in Freund's incomplete adjuvant, and after another one week, 10μg of the protein was injected into the abdominal cavity. The mice were bled one week after the final booster.

*Immunocytochemistry*

Infected muscles of 42-day post infection were fixed with 4% paraformaldehyde and embedded in OCT compound (Miles Inc., Elkhart, Indiana USA) and frozen. Cryostat sections (4 μm in thickness) were transferred to albumin coated microscope slides. The sections were immunostained using Histostain SP kit (Zymed Laboratories Inc., San Francisco, California USA) as described by the manufacturer's instructions. Briefly, the sections were treated with the first antibodies (×100 diluted antibody against the NBL21 fusion protein or control normal mouse serum) for 30 min at room temperature. After washing, the sections were treated with the biotinylated second antibody (Zymed Laboratories Inc.) for 30 min at room temperature, washed three times, treated with avidin coupled with peroxdase (Zymed Laboratories Inc.) for 10 min at room temperature, and reacted with diaminobendizone solution (Zymed Laboratories Inc.) for peroxidase staining.

*Antigenicity analysis of the fusion protein NBL21*

The fusion protein NBL21 was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to nitrocellulose membranes. Membranes were blocked with skim milk and reacted with the first antibodies (×100 diluted) including anti NBL antibodies, infected sera, antibodies against excretory and secretory (ES) products of muscle larvae, and control sera for 60 minutes at 37 ℃. After washing, the membranes were treated

with alkaline phosphatase-conjugated anti-mouse IgG for 30 min at 37℃. The alkaline phosphatase was developed by using BCIP and NBT.

*Specificity analysis of antibody against the fusion protein NBL21*

Reactivity of anti-NBL21 fusion protein antibody was checked against 4 kinds of antigens including crude antigens and ES products from the two species (*T. spiralis* and *T. pseudospiralis*). The antigens were subjected to SDS-PAGE and reacted to the anti NBL21 antibodies (×100 diluted) for 60 min at 37℃. The nitrocellulose membranes were treated in the same way as described above.

*Specificity of fusion protein NBL21*

Antigen specificity of the fusion protein was assessed in two ways. The panel of crude antigens of parasites (*Dirofilaria immitis, Ascaris lumbricoides, Toxocara canis, Gnathostoma nipponicum, Sparganum mansoni, Clonorchis sinensis, Anisakis simplex, Strongyloides stercoralis, Paragonimus westermani, Paragonimus miyazakii, Fasciola hepatica* and *Cysticercus cellulosae*) was subjected to reaction against anti fusion protein NBL21 antibody by multiple dot ELISA according to the established method. The other method included ELISA where fusion protein NBL21 was reacted against a panel of human patient sera (trichinosis, paragonimiasis, dirofilariasis, dihyllobothriasis, anisakiasis, cysticercosis cellulose hominis, trichuriasis, gnathostomiasis and fasciolasis) according to the established method.

*Kinetics of antibody response against the fusion protein NBL21*

Three BALB/c mice were each inoculated orally with 300 infective larvae. Sera from infected mice were collected before inoculation for a control and at 1, 2, 3, 4, 6, 8 and 10 weeks after inoculation. Antibody titer against fusion protein NBL21 was assessed by means of ELISA according to the established method.

*Nucleotide sequence accession number*

The nucleotide sequence reported in this article has been submitted to the GenBank database and has the accession number AY033438.

## RESULTS

*DNA and amino acid sequences*

The cDNA library from NBL gave 66 positive clones, which comprised of 3 groups of clones. One clone, designated NBL21, from the major group was further subcloned. The clone NBL21 consisted of 1656 bp nucleotide and encoded 552 amino acid residues (Fig. 1). A homology study from NCBI BLAST search revealed significant homology with myosin heavy chain of some nematodes including *Caenorhabditis elegans* (GenBank under accession number X08067), *Onchocerca volvulus* (GenBank under accession number M74066) and *Brugia malayi* (GenBank under accession number L01625).

Myosin heavy chain of *C. elegans* had 65% homology in the DNA sequence and 66% in the deduced amino acid sequence. Myosin heavy chain of *O. volvulus* had 64% homology in the DNA sequence and 63% in the deduced amino acid sequence. Myosin heavy chain of *B. malayi* had 62% homology in the DNA sequence and 64% in the deduced amino acid sequence. In some areas such as amino acid sequence 260-293 (box in Fig. 1), more significant homology was found; 75-77% homology in the DNA sequence and 88-94% in the deduced amino acid sequence.

*Western blotting*

The fusion protein NBL21, produced by means of an *E. coli* expression system, was migrated at 68 kDa on SDS-PAGE and positively immunostained with anti *T. spiralis* NBL antibody (arrow in lane 1 of Fig. 2), *T. spiralis* infected sera (arrow in lane 2 of Fig. 2), *T. pseudospiralis* infected sera (arrow in lane 3 of Fig. 2), but not with anti ES product of *T. spiralis* muscle larvae (lane 4 in Fig. 2), anti ES product of *T. pseudospiralis* muscle larvae (lane 5 in Fig. 2) nor with control sera (figure not shown).

An SDS-PAGE analysis revealed that crude antigens and ES product of muscle larvae were composed of many bands with different molecular weights. Out of many bands, only one band of 200 kDa (corresponding to the expected size) of crude antigens of *T. spiralis* (lane 2 in Fig. 3) and *T. pseudospiralis* (lane 4 in Fig. 3) were positive by immunostaining with the antibody against NBL21 fusion protein. No bands of the ES product of the two species were positive by the immunostaining (lane 3, 5 in Fig. 3).

*Immunolocalization*

As shown in Fig. 4 A, strong positive immunostaining was observed in the internal structures of the muscle larvae. The staining consisted of a spotty pattern and lining beneath the cuticle surface, and internal organs were negative by the staining. The control sera failed to stain any structures (Fig. 4 B).

*Antigen specificity*

The multiple dot ELISA showed reactivity of the antibody against the fusion protein NBL21 against crude extracts of *D. immitis, A. lumbricoides, T. canis, G. nipponicum, S. mansoni, C. sinensis* but not against *A. simplex, S.*

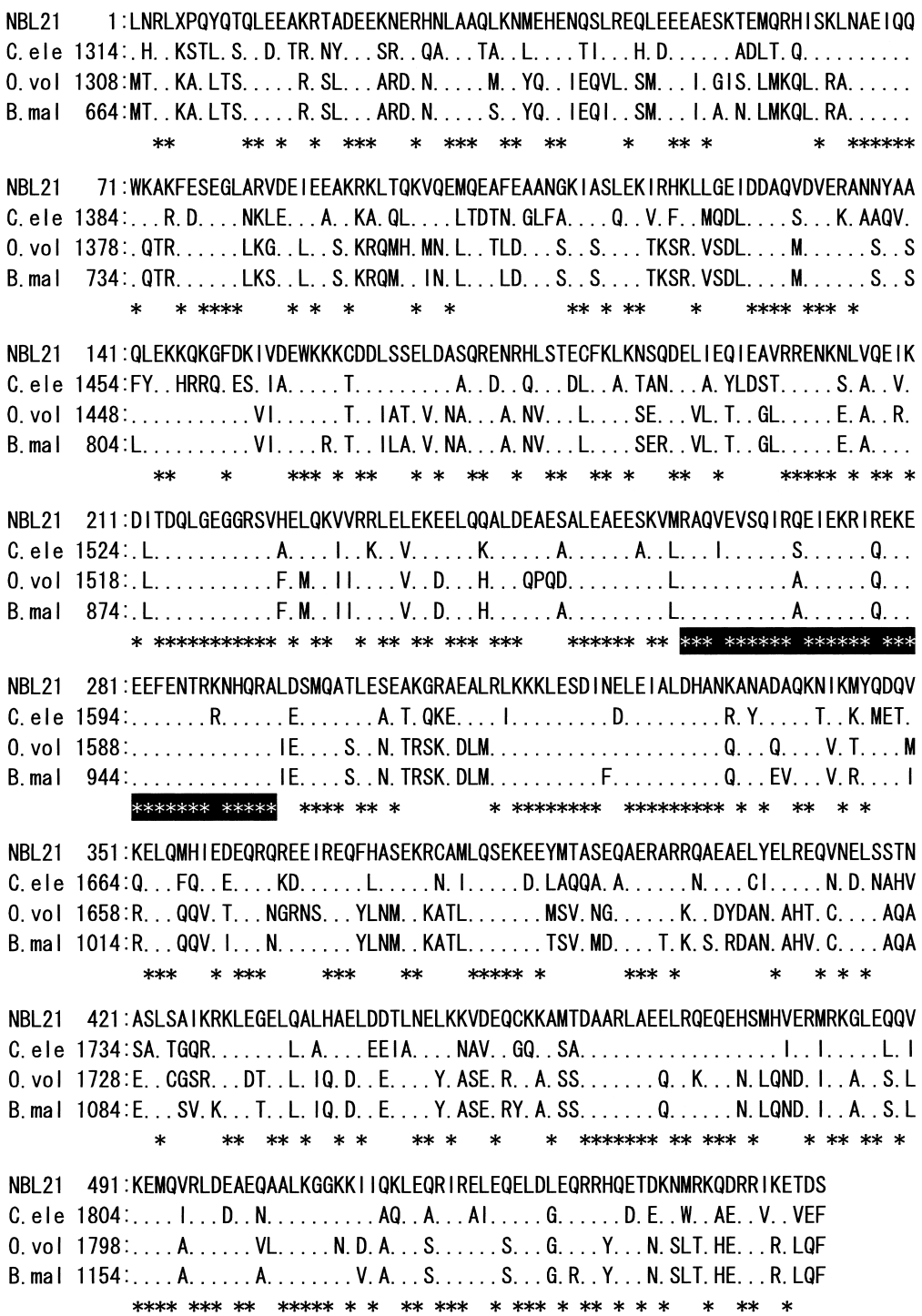


Figure 1 Comparison of a deduced amino acid sequence of the clone NBL21 and its comparable genes from other nematodes including *C. elegans* (C. ele; amino acid residues 1314-1865 in the total 1969 residures), *O. volvulus* (O. vol; amino acid residues 1308-1859 in the total 1957 residues), *B. malayi* (B. mal; amino acid residues 664-1215 in the total 1313 residues). The asterisks indicate nucleotides, which are identical to those of C. ele, O. vol, B. mal and NBL21. A box shows high homology region mentioned in the text.

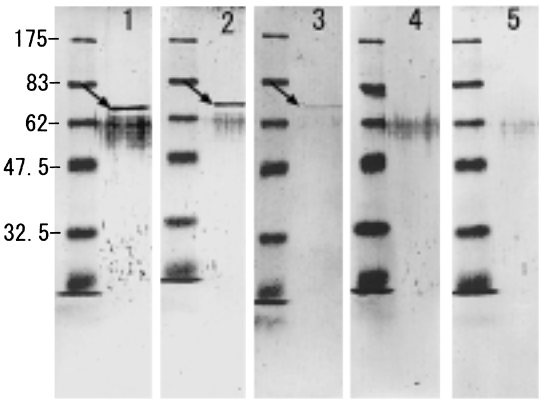


Figure 2 Western blots analysis of the fusion protein NBL21. *E. coli* expressed NBL21 fusion protein was reacted with anti NBL21 antibody (lane 1), infected sera of *T. spiralis* (lane 2), infected sera of *T. pseudospiralis* (lane 3), anti ES product of *T. spiralis* antibody (lane 4) and anti ES product of *T. pseudospiralis* antibody (lane 5). The left side of each lane is molecular marker and the right side is the test sample. The numbers of the left side is molecular weight. The arrow shows positive band.

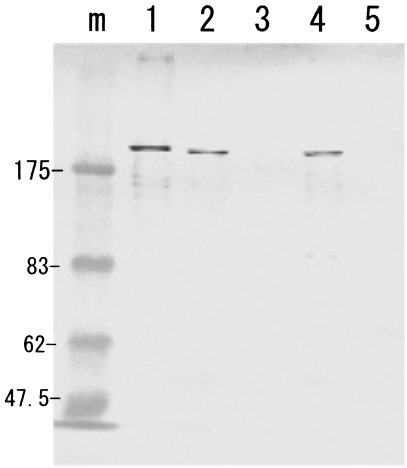


Figure 3 Western blot analysis of reactivity of anti NBL21. The mouse antibody produced against the fusion protein NBL 21 was reacted with NBL crude antigen (lane 1), *T. spiralis* crude antigen (lane 2), *T. pseudospiralis* crude antigen (lane 4), ES products of *T. spiralis* (lane 3) and ES products of *T. pseudospiralis* (lane 5). The numbers are molecular weights of marker (m).

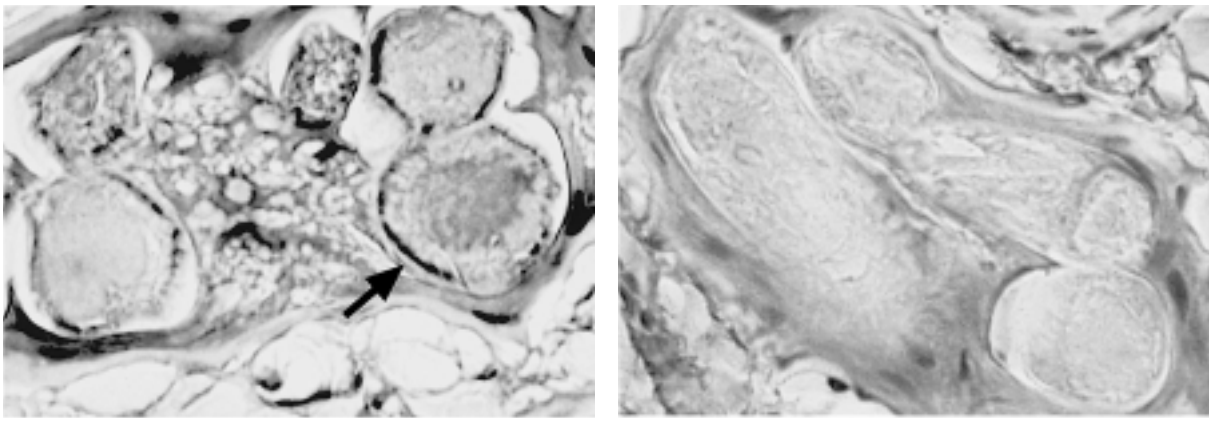

Figure 4 Immunocytochemical staining of muscle larvae of *T. spiralis* with antibody against NBL 21 fusion protein (A) and control serum (B). The arrow shows the positive staining.

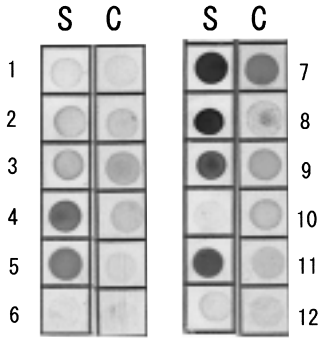


Figure 5 Dot - ELISA analysis of reactivity of anti NBL21 fusion protein antibody (S) and normal mouse sera (C) against a panel of crude antigens. 1. *P. westermani*, 2. *P. miyazakii*, 3. *F. hepatica*, 4. *C. sinensis*, 5. *S. mansoni*, 6. *C. cellulosae*, 7. *D. immitis*, 8. *T. canis*, 9. *A. lumbricoides*, 10. *A. simplex*, 11. *G. nipponicum*, 12. *S. stercoralis*. Crude antigens from *C. cellulosae*, *A. simplex* and *S. stercoralis* gave negative results which may suggest major difference in antigenic epitope of myosin heavy chain from other parasites.

*stercoralis, P. westermani, P. miyazakii, F. hepatica* and *C. cellulosae*. *D. immitis* and *T. canis* especially exhibited strong reactivity ("S" in Fig. 5). Normal mouse sera ("C" in Fig. 5) gave negative or weak positive result.

The fusion protein reacted positive for ELISA against sera from all patients examined including nematode, cestoide and trematode infection (figures not shown).

*Antibody response kinetics*

Antibodies against the fusion protein were detected from 4 weeks after the infection and reached a maximum at 8 weeks, and the ELISA titer remained high throughout the 10-week-long experiment (figures not shown).

## DISCUSSION

Recent progress in cell biology has enabled us to produce a large amount of proteins with high purity. Taking advantage of this methodology, proteins of a parasite origin have been mass-produced for a variety of usages including immunodiagnostic antigen (Chung and Ko, 1999) and vaccine (Sun *et al*., 1994; Prema *et al*., 1994). Genes encoding specific antigens of ES products (43, 39 and 53 kDa) have been characterized (Sugane and Matsuura, 1990; Zarlenga and Gamble, 1990; Su *et al*., 1991; Vassilatis *et al*., 1992).

In this study we obtained the clone NBL21 from *T. spiralis* NBL. This clone is likely a part of the myosin heavy chain from the sequence homology and in situ localization of this protein within the parasite. This study also revealed some basic profiles of the myosin heavy chain including the DNA and amino acid sequence which is discussed with comparable genes of other nematodes such as *C. elegans*, *B. malayi* and *O. volvulus*. Antigenic specificity and possible immunodiagnostic usages were also assessed.

Myosin is a fundamental protein shared by all kinds of cells. The DNA sequence of such a protein should be highly conservative. This notion is supported by the present study which showed high homology among other nematodes. In particular, some areas of the nucleotides are significantly conserved. Such area may be responsible for its common function as myosin.

The peptide NBL21 seems to carry immunodominant antigen which is recognized by hosts during the experimental infection. A similar result was reported by Li *et al*. (1995) who demonstrated that myosin heavy chain carries immunodominat filarial antigen. The clone NBL21 had 48% homology with the myosin heavy chain of hosts (humans and mouse), unshared sequence may explain the reasons why the fusion protein NBL21 is highly antigenic against the hosts.

We have no ready explanation as to how this internal antigen is exposed to the host immune system. Interestingly, paramyosin, which is also an internal constituent of parasites, carries immunodominant antigen which may be used as a protective antigen against schistosome infection (Xu *et al*., 1989). Our preliminary experiment, however, showed that the fusion protein NBL21 does not provoke protective immunity against challenge infection.

Fusion proteins of parasites can be a candidate for immunodiagnostic antigen as suggested by many authors (Zarlenga and Gamble, 1990; Chandrashekar *et al*., 1991; Zarlenga *et al*., 1994). The mice infected with *Trichinella* seem to provoke antibodies at detectable levels against the fusion protein NBL21 from the early phase of the infection, therefore, the antigen of the fusion protein NBL21 seems to be Group I antigen (Denkers *et al*., 1990) and rapid responding group antigen (Takahashi *et al*., 1990). One drawback of this fusion protein is its lack of species specificity. The antibody against the fusion protein NBL21 reacted against sera from a variety of parasite crude antigens. Therefore, this fusion protein can be used for early diagnosis of some parasite infection.

## ACKNOWLEDGEMENTS

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# DRUG SUSCEPTIBILITIES AND CLINICAL MANIFESTATIONS OF *MYCOBACTERIUM TUBERCULOSIS* IN ZAMBIA

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**Abstract**: The inappropriate use of anti-tuberculosis drugs has resulted in an increase in the drug resistance. This study was conducted to obtain data related to the spectrum of drug resistant *Mycobacterium tuberculosis*. A total of 499 strains of *M. tuberculosis* were tested for susceptibilities of isoniazid (INH), rifampicin (RFP), ethambutol (EMB) and streptomycin (SM). Totally 225 patients were also evaluated for their clinical manifestations. The primary and acquired resistances were observed in 13.9% and 16.1% to INH, 11.1% and 12.9% to RFP, 6.7% and 9.7% to EMB, and 3.8% and 3.2% to SM respectively. There were no significant differences between primary and acquired resistances. Multi-drug resistance was observed in 22 strains (4.4%). Clinical manifestation, as measured by chest roentgenograms, was similar in patients with either drug-susceptible or resistant strains. The clinical and radiological information was not useful for the prediction of susceptible and resistant strains. The HIV serostatus was not associated with drug susceptibility profiles either. Our study has documented a high incidence of drug resistant *M. tuberculosis*, with no obvious clinical correlates, which must be considered when implementing a strategy for chemotherapy. As for clinical practices, many defaults during chemotherapy reduced the treatment successes. It is important to assess the efficient implementation of the directly observed treatment with short course chemotherapy (DOTS) and interrupt the further dissemination of resistant *M. tuberculosis* in the community. The drug resistances must be surveyed continuously to obtain useful clinical perspective and evaluate the effectiveness of tuberculosis control program.
**Key words**: Resistance, Multi-drug, Primary, Acquired, Zambia

## INTRODUCTION

The multi-drug resistant *Mycobacterium tuberculosis* (MDRTB) infection is one of the major problems for tuberculosis control and treatment. In 1993, the emergence of life-threatening MDRTB in New York City among AIDS patients was reported (Frieden *et al*., 1993). AIDS patients are susceptible to repeated episodes of new tuberculosis infections. Administration of anti-tuberculosis chemotherapy results in a high probability of evolution of MDRTB strains within the community of HIV infected individuals with weakened immune systems (Gordin *et al*., 1996 and Spellman *et al*., 1998).

The prevalence of tuberculosis in Sub-Saharan African countries has increased in these 10 years. In Zambia, it is approximately 500 per 100,000 people and 40,417 new patients were recorded in 1996 (WHO report, 1998). In Zambia, 73% of the adult tuberculosis patients are HIV-1 seropositive and 13% of all adult deaths are due to tuberculosis. With HIV epidemic, tuberculosis is a major cause of death

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(ZAMBART, 1999; Elliott *et al*.,1996). The shortages of both medical resources and knowledge about the disease are impediments to adequate treatment of patients, resulting in an increase in drug resistant *M. tuberculosis* (Bosman, 2001).

For effective treatment of tuberculosis, anti-tuberculosis drug susceptibility against the pathogen is essential information. The national anti-tuberculosis program in Zambia has not been operative since 1997. From that time, the country has not produced the national figures for tuberculosis control. The goal of this study was to obtain updated information on the drug susceptibility status of *M. tuberculosis* infections in Zambia. The readily recognisable clinical manifestations of tuberculosis infection were also evaluated for their possible relationship to organism drug susceptibility.

## MATERIALS AND METHODS

*Definitions of terms*

INH, RFP, EMB, and SM were stipulated as firstline drugs for treatment of tuberculosis. New case is a patient who has never had treatment for tuberculosis, or who has taken anti-tuberculosis drugs for less than 4 weeks. Retreatment case is a patient who was treated previously and requiring retreatment due to relapse, default and treatment failure. Primary drug resistance is the presence of resistant strains of *M. tuberculosis* in a patient with no history of prior treatment. Acquired drug resistance is the resistance found in a patient who had previously received at least one month of anti-tuberculosis treatment, as documented in the tuberculosis registry, or medical records or by the patient's account. Mono resistance is a presence of resistance to single drug. Combined drug resistance is the prevalence of drug resistance among all cases of tuberculosis, regardless of treatment history. Any resistance is the prevalence of drug resistance including one designated drug at least. For example, In the case of INH, it is a total of resistant strains to INH, INH+RFP, INH+EMB, INH+SM, INH+RFP+EMB, INH+RFP+SM, INH+EMB+SM, and INH+RFP+EMB+SM.

*Clinical specimens*

Sputum specimens were collected at the Chest Clinic in the University Teaching Hospital (UTH) in Lusaka, Zambia between March, 1999 and August, 2000. The UTH is the third referral hospital located in the capital city of Zambia. Patients are referred from the entire Zambia. Suspected tuberculosis patients who visited the Chest Clinic were requested to submit consecutive three sputum specimens for laboratory examinations. The specimens are sent to the UTH laboratory for routine fluorescent smear examination. The sputum specimen yielding a positive smear result was cultured on conventional Lewenstein-Jensen media. The patients with positive smear were enrolled randomly to the study under informed consent and subjected to radiological examinations. Some sputum specimens only for drug susceptibility testing were collected from routine laboratory specimens. Blood specimens for haematological and immunological examination were collected from randomly selected tuberculosis patients.

*Smear, culture and drug susceptibility testing*

One portion of purulent sputum specimen was stained with auramine-phenol method and examined under a fluorescence microscope (200×). The smear positive sputum specimen was cultured on Lewenstein-Jensen medium containing glycerol (L-J G) or pyruvate (L-J P). The inoculated tubes were incubated at 37℃ and inspected weekly for up to 8 weeks. The isolates were subjected to Ziehl-Neelsen staining for acid-fastness, niacin accumulation, *p*-nitrobenzoic acid (PNBA) sensitivity, thiophen-2-carboxylic acid hydrazide (TCH) and growth at 25 and 42℃ for identification of *M. tuberculosis*. Susceptibility testing to isoniazid (INH), rifampicin (RFP), ethambutol (EMB), and streptomycin (SM) (Sigma-Aldrich Company Ltd., Dorset, UK) was performed using resistant ratio method on L-J G medium. The resistance ratio is defined as the minimum inhibitory concentration (MIC) of the test strain divided by the MIC of the standard susceptible *M. tuberculosis* strain in the same set of tests. A resistance ratio of 2 or less defines drug susceptibility, while 4 or more is considered evidence of drug resistance. The concentrations for each drug were as follows: INH 0.05, 0.1 and 0.2 $\mu$g/m*l*; RFP 12.5, 25 and 50 $\mu$g/m*l*; EMB 0.8, 1.6 and 3.2 $\mu$g/m*l*; SM 10, 20 and 40 $\mu$g/m*l*. Wild standard strains for control were obtained from the Chest Disease Laboratory (the national reference laboratory in Zambia).

Part of the specimen was also evaluated using the proportion method. The ratio between the number of colonies on drug-containing medium and the number of colonies on drug-free medium indicates the proportion of drug resistant *M. tuberculosis* present in the population. From these bacterial colony counts, the proportion of resistance can be determined and as a percentage of the total number of viable colony forming units in the population. Below a certain fraction, called the "critical proportion", a strain is classified as susceptible; above that, as resistant. The critical proportion for resistance was taken as 1% in general. The critical drug concentrations were as follows: INH 0.2 $\mu$g/m*l*; RFP 40 $\mu$g/m*l*; EMB 2.0 $\mu$g/m*l*; SM 10 $\mu$g/m*l*.

*Clinical data*

All patients were queried as to whether they had been previously treated for tuberculosis. Additionally chest roent-

genograms were evaluated for opacity, cavity, pleurisy and lymphadenopathy. Patients were selected randomly for HIV evaluation. All patients were enrolled with informed consent and given pre-and post-testing counsel. Blood specimens were obtained for both HIV antibody testing (BIONOR HIV-1&2: BIONOR, Norway) and CD4/CD8 analysis by flow cytometry (Becton-Dickinson, California, USA).

*Statistical analysis*

Statistical analysis was done using chi-square or Fisher's exact test. A p value of less than 0.05 was considered significant.

*Ethical consideration*

The study protocol has been approved by the ethics committee of the University of Zambia. Subjects were enrolled after informed consent. Subjects younger than 15 years were excluded from the study.

## RESULTS

*Drug susceptibility*

A total of 499 strains of *M. tuberculosis* were examined for susceptibility to 4 firstline anti-tuberculosis drugs. Out of 499 strains, 257 were isolated from sputum specimens of study-enrolled patients. The remaining 242 were from sputum specimens for routine diagnosis. Only information about sex and age were available for these 242 specimens. In total, there were specimens from 271 males and 228 females. The mean age was 31.7±10.3 years old. A total of 365 (73.1%) patients were under 40 years old. Sixty strains to INH (12.0%) and 52 to RFP (10.4%) were resistant respectively. Mono and any resistance to INH were observed in 4.6% and 12.0% in combined drug resistance evaluation. In the same way, mono and any resistance were observed in 5.2% and 10.4% to RFP, 2.4% and 7.2% to EMB, and 1.6% and 3.8% to SM respectively. The primary and acquired resistances were observed in 13.9% and 16.1% to INH, 11.1% and 12.9% to RFP, 6.7% and 9.7% to EMB, and 3.8% and 3.2% to SM respectively. The multi-drug resistance was observed in 22 strains/cases (4.4%). The primary, acquired and combined resistances are summarised in Table 1. There were no significant differences between primary and acquired resistances. Twelve strains (2.4%) were resistant to 3 drugs or more.

A total of 297 of the 499 strains were also evaluated with the proportion method. There were incidences of combined drug resistance of 11.7% to INH, 9.1% to RFP, 7.5% to EMB, and 10.4% to SM. There was statistically significant difference between resistant ratio and proportion method for the analysis of SM susceptibility (p=0.0004).

*Clinical data*

A total of 509 chest roentgenograms were available for a comparison of new and retreatment cases. The only difference observed between the two groups was cavity formation (p=0.019). There were no differences in the clinical manifestations when data from 169 drug-susceptible patients were compared to those from 56 drug resistant patients (Table 2).

Patients were treated with short course chemotherapy for the standard 8 months period using the combination of INH, RFP, EMB and pyrazinamide (PZA). Of 509 patients 394 among 433 new cases (91.0%) received this combination therapy while 29 (38.2%) for 76 retreatment cases (Table 3). The WHO standard for retreatment case was 5-drug combination with INH, RFP, EMB, PZA and SM.

During the same study period, clinical outcomes of tuberculosis patients were monitored for 461 cases including study population. A total of 225 patients (48.8%) achieved negative sputum conversion at the time of 8-month while 152 patients (33.0%) defaulted in total. The 8-month mortality of retreatment cases was no higher than of new cases (Table 4).

Fifty-two blood specimens were obtained from ran-

Table 1 Drug susceptibility patterns of primary, acquired and combined resistance

| | Primary (%) | Acquired (%) | Combined (%) |
|---|---|---|---|
| Number of strains | 208 | 31* | 499 |
| Mono resistance | | | |
| INH (mono) | 9 ( 4.3) | 2 ( 6.5) | 23 ( 4.6) |
| RFP (mono) | 11 ( 5.3) | 1 ( 3.2) | 26 ( 5.2) |
| EMB (mono) | 5 ( 2.4) | 1 ( 3.2) | 12 ( 2.4) |
| SM (mono) | 3 ( 1.4) | 0 ( 0.0) | 8 ( 1.6) |
| Resistant to 2 drugs | 17 ( 8.2) | 3 ( 9.7) | 29 ( 5.8) |
| Resistant to 3 drugs | 4 ( 1.9) | 1 ( 3.2) | 8 ( 1.6) |
| Resistant to all 4 drugs | 0 ( 0.0) | 0 ( 0.0) | 4 ( 0.8) |
| Any resistance | 49 (23.6) | 8 (25.8) | 110 (22.0) |
| Multi-drug resistance | 11 ( 5.3) | 2 ( 6.5) | 22 ( 4.4) |

*18 of 257 patients had no data about the duration of preceding treatment.

Table 2 A comparison of clinical manifestation

| Category | No. of patients (male/female) | Mean age±SD | Chest roentgenogram findings (%) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | FA | MO | MI | Cavity | LYM | Pleurisy |
| New | 433 (247/186) | 31.7±10.1 | 74 (17.1) | 229 (52.9) | 130 (30.0) | 230 (53.1) | 25 (5.8) | 51 (11.8) |
| Retreatment | 76 ( 42/ 34) | 31.2± 9.0 | 17 (22.4) | 42 (55.2) | 17 (22.4) | 52 (68.4)* | 1 (1.3) | 13 (17.1) |
| Susceptible | 169 ( 89/ 80) | 31.8±11.0 | 36 (21.3) | 88 (52.1) | 45 (26.6) | 93 (55.0) | 11 (6.5) | 22 (13.0) |
| Resistant (any) | 56 ( 37/ 19) | 32.4± 9.7 | 11 (19.6) | 29 (51.8) | 16 (28.6) | 36 (64.3) | 1 (1.8) | 8 (14.3) |

FA = Far advanced: MO = Moderate: MI = Minimal: LYM = Hilar lymphadenopathy
* p = 0.019

Table 3 Treatment regimen of tuberculosis patients

| | Number of patients (%) | |
|---|---|---|
| | New cases | Retreatment cases |
| Initial treatment | 433 | 76 |
| INH/RFP/EMB or SM/PZA | 394 (91.0) | 30 (39.5) |
| INH/RFP/EMB/PZA/SM | 6 ( 1.4) | 42 (55.2) |
| INH/RFP/PZA | 12 ( 2.8) | 0 ( 0.0) |
| INH/RFP/EMB | 1 ( 0.2) | 0 ( 0.0) |
| RFP/EMB/PZA | 1 ( 0.2) | 0 ( 0.0) |
| RFP/PZA | 2 ( 0.5) | 0 ( 0.0) |
| No record | 17 ( 3.9) | 4 ( 5.3) |

Table 4 Clinical outcomes of tuberculosis patients

| | Number of patients (%) | |
|---|---|---|
| | New cases | Retreatment cases |
| Outcome at 8 month | 444 | 17 |
| Sputum smear | 251 (56.5) | 9 (52.9) |
| Positive | 9 ( 2.0) | 0 ( 0.0) |
| Negative | 216 (48.6) | 9 (52.9) |
| Not done | 26 ( 5.9) | 0 ( 0.0) |
| Default | 145 (32.7) | 7 (41.2) |
| Dead | 42 ( 9.5) | 1 ( 5.9) |
| Transferred | 6 ( 1.3) | 0 ( 0.0) |

domly selected pulmonary tuberculosis patients. The HIV-seropositive rate was 69.2% (36/52 patients). Within the 36 HIV-seropositive patients, 34 were available for haematological testing. The mean CD4 lymphocyte number was 375.0±307.9/$\mu$l in total. Totally 35.3% (12/34) had less than 200/$\mu$l CD4 lymphocytes. There was no statistical difference in the drug susceptibility to 4 firstline drugs between HIV positives and negatives.

## DISCUSSION

In the present study, the prevalence of anti-tuberculosis drug resistance in Lusaka, Zambia was high and represents a major problem for national tuberculosis control. The high incidence of resistant *M. tuberculosis* in Africa has been documented in several studies. Weyer and Kleeger (1992) reported in the 1980's that the initial resistance in South African adult black patients was 14.2% to INH, 12.1% to SM, 1.8% to RFP and 2.5% to EMB respectively. The incidences, by 1995, in Ivory Coast were 11.3%, 6.9%, 5.3% and 0.3% respectively (Pablos *et al*., 1998). The multi-drug resistance was reported less than 1% in 1993 in Zambia (Chest Disease Laboratory, 1994), but mono resistance to INH and RFP, as reported from the Chest Disease Laboratory, was 4.7% and 1.8% by 1994. In the absence of a national tuberculosis control program, the drug resistance incidence has increased in recent years.

There are several possible explanations for rising drug resistance. One possible reason is an irregular administration of anti-tuberculosis drugs. It was shown that general clinicians were following standard combination chemotherapy to treat tuberculosis patients although the Zambian government has not adopted DOTS as a national strategy. However, the treatments were completed in only 56.5% of the patients with a 32.7% default rate among new cases. Additionally, the mortality during treatment was 9.5%. An unstable drug supply and insufficient awareness in tuberculosis treatment may be the cause of low treatment completion. The anti-tuberculosis drugs are provided by the government without cost for tuberculosis patients in Zambia. However, the supply is neither constant nor sufficient resulting in insufficient treatment at clinics. The shortage of drugs in the health institutions discourages patients from coming to the hospital and health clinics. When supplies failed, the purchase of drugs was beyond the financial means of most patients. It is also conceivable that many patients defaulted from treatment because transportation to the clinic was either difficult or unavailable.

Another possible reason is relapse or re-infection with resistant strains. HIV prevalence in Zambia is approximately 20% in antenatal women (Handema *et al*., 2001). The epidemic has a potential for the development of *M. tuberculosis* drug resistance among HIV-infected individuals as a result of the increased frequency of drug resistance including MDRTB in the population (Bradford *et al*., 1996). However, as indicated in this study, the frequency of drug

resistance was not significantly different between HIV positives and negatives. Even for the retreatment cases the frequency of resistance was not higher than new cases. The data suggest that the resistant strains are spreading within the community and causing new infection in the population rather than evolving *de novo* from each patient.

Multi-drug resistance was observed in 4.4% of total isolates. Even with this high MDRTB frequency, conversion to negative sputum at 8 month was achieved in 86.1% (216 of 251 evaluated cases) of the new patients with appropriate treatment. Although the impact of MDRTB on the clinical situation at individual level is severe (Goble *et al*., 1993; Mitchison and Nunn, 1986), it may not require any revision to short course chemotherapy regimen as a national strategy. The blind use of a 5-drug combination for retreatment cases may not be efficient, even though that is the recommendation of the WHO (WHO, 1996). The frequencies of drug resistance were not significantly different between primary and acquired cases. Additionally, most of the patients were new cases. In this situation, it is not informative to distinguish new from retreatment cases, but rather to follow the patient, irrespective of drug resistance status, for treatment efficacy. The suspected resistant patient who shows no negative conversion of sputum will be transferred to an appropriate referral hospital for further treatment even with second and third line drugs.

A clinical indicator of drug-resistant *M. tuberculosis* infection would be useful because it is impractical to perform culture and drug susceptibility examinations for each patient in Zambia. As mentioned above, the clinical history was not useful to predict resistance. The chest roentogenogram is quick and feasible examination even in developing countries. However, it was unable to distinguish between drug-susceptible and drug-resistant disease. Also the HIV-serostatus were not informative for the prediction of drug resistances in the present study. Some techniques for the detection of resistance using molecular methods have developed in advanced countries (Abe *et al*., 2000), but these may be beyond the resources of developing countries. Thus, the search for a clinical or physical finding that correlates to drug susceptibility must continue.

In the present study, the resistant ratio method was employed for drug susceptibility testing. The methods used for the definition of drug resistance did not appear to be critical although the result figures for SM were different between resistant ratio and proportion method. The difference observed for SM between resistant ratio and proportion method was likely due to the critical drug concentrations employed for examination. The minimal drug concentration for resistant ratio method was 10 $\mu$g/m*l* and the critical concentration for proportion method was also 10 $\mu$g/m*l*. Taking this concentrations into consideration, if the strains indicating ratio of 2 were taken as resistant, the frequency became 9.8% as compared to 10.4% using proportion method. In this setting, the difference was not significant. However, it will be important to introduce proportion method widely in Zambia for data generalisation.

Tuberculosis has risen together with HIV epidemic. As it is shown in this study, approximately 70% of tuberculosis patients were HIV-seropositive. However, even in the HIV-infected patients, approximately 65% showed above 200/$\mu l$ CD4 lymphocytes. It was almost identical to other reports (Elliott *et al*., 1995; Kassim *et al*., 1995). Additionally, only 4 patients (11.8%) had less than 100/ $\mu l$ CD4 lymphocyte. Good treatment results are expected even in HIV-infected patients with tuberculosis in Zambia despite the difficulty in curing the severely immunocompromised patient.

The treatment outcome in the present study was far from satisfactory. It was not a direct objective of this study to increase treatment success. However, the treatment of tuberculosis was not efficient because of poor cure rate and many defaults over 30% both in new and retreatment cases. For reducing treatment failure and default, the DOTS strategy is proven to be effective (WHO report, 2001). As reported in Korea, the prevalence of drug resistance can be reduced with proper treatment (Hong *et al*., 1998). Therefore, it is important to implement DOTS or DOTS like confident treatment efficiently and monitor drug resistance consecutively. If drug resistant infection is detected, it will be required to employ DOTS-Plus strategy that uses individualised treatment regimens with second-line anti-tuberculosis drugs.

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# PROCEEDINGS OF THE 42ND ANNUAL MEETING OF JAPANESE SOCIETY OF TROPICAL MEDICINE

22-24 September 2001, Tokyo

**President**

Masamichi Aikawa
Professor, Research Institute of Science and Technology
Tokai University

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B-13 Survey on patients with imported infectious deseases admitted to the Department of Infectious Deseases Ohnishi, K. and Kato, Y.

B-14 Current status of imported malaria in Japan since April, 1999 Ohtomo, H. *et al.*

B-15 Analysis of malaria surveillance data based on infectious disease prevention law in Japan Kaku, K. *et al.*

B-16 How could the ISTM's mailing list Travel Med be utilized? Kimura, M. *et al.*

B-17 Cerebral metabolic changes in a primate model of severe human malaria as studied by positron emission tomography Sugiyama, M. *et al.*

B-18 Glucose metabolic change of spleen in malaria-infected *Macaca fuscata*: correlation of FDG-PET results with pathological findings Ikeda, E. *et al.*

B-19 Cerebral ring hemorrhages in squirrel monkeys infected with *Plasmodium falciparum* Katakai, Y. *et al.*

B-20 Identification of malarial antigen-mimicking peptides Ishikawa, D. *et al.*

B-21 Construction of anti-*P. falciparum*/anti-CD3 bispecific scFv with antiparasiticidal function Yoshida, S. *et al.*

B-22 *Plasmodium vivax* transmission-blocking vaccine: efficacy study on human isolates Tsuboi, T. *et al.*

B-23 An attempt to identify genes controlling susceptibility to mouse severe malaria Nagayasu, E. *et al.*

B-24 Biochemical analysis of complex Ⅱ from *plasmodium falciparum* mitochondria Mi-Ichi, F. *et al.*

B-25 Molecular characterization of Al-cys peroxiredoxin from the human malaria parasite *Plasmodium falciparum* Kawazu, S. *et al.*

B-26 Effect of Minocycline in combination with antimalaria drug on the elimination of *Plasmodium berghei* and chloroquine resistant-*P. chabaudi* parasites in mice Hando, K. *et al.*

B-27 Anti-malarial effects of dipyridamole *in vitro* and *in vivo.* Nakano, Y. *et al.*

B-28 Development of new antimalarial drug-*in vitro* and *in vivo* antimalarial activity of endoperoxides Ono, K. *et al*.

B-29 A public/private partnership among the Japanese pharmaceutical industry, the government and WHO/TDR (JPMW) : A research project for discovery of new antimalarial drugs in Japan Hata, K. *et al.*

B-30 Both mosquito-derived xanthurenic acid and a host blood-derived factor regulate gametogenesis of *Plasmodium* in the midgut of the mosquito Arai, M. *et al.*

B-31 Studies on malaria micro-gemetocyte exflagellation inducing factor in the salivary gland of *Anopheles stephensi* Matsuoka, H. *et al.*

President's lecture

# THE PATHOLOGY OF MALARIA

MASAMICHI AIKAWA

Research Institute of Science and Technology, Tokai University

Study of the pathology of malaria has contributed to understanding of its pathogenesis. I will summarize my work of 30 years in the fields of malaria pathology as follows. The main pathologic changes appear to be caused by the response of the reticuloendothelial system to the parasaite, by the destruction of erythrocytes which together with their defective production causes anemia and by the obstruction of capillary lumens in the brain and other organs by parasitized erythrocytes (PRBC). Most pathologic studies of malaria are of *P. falciparum* which causes severe malaria. Cerebral malaria is recognized by the blockage of the cerebral capillaries by PRBC and associated pathologic lesions. Based on these findings, the development of malaria vaccines against cerebral malaria is now in progress. Splenomegaly and hepatomegaly are common findings among malaria patients. Renal disease also occurs in malaria. In falciparum malaria, mesangiopathic glomerulonephritis causes abnormal urinary sediment including proteinuria and cylinduria. In contrast, with quartan malaria membrano-proliferative glomerulonephritis leads to a nephrotic syndrome. In pregnancy, falciparum malaria severely affects the placenta, especially in primiparous women, often resulting in abortion. Microscopic fields show intervillous spaces with many PRBC and macrophages. Recently adhesion molecules such as hyaluronic acid and CSA have been identified. These molecules assist PRBC to cytoadhere to trophoblasts and PRBC sequestration results.

Despite the fact that both the anatomic and microscopic alterations associated with malaria are well recognized, the pathophysiologic changes in malaria infection are not well characterized. A better understanding of the mechanisms of malaria infection may contribute to more efficacious treatment with pharmacological, vaccines and molecular approaches.

Key note speach (lecture)

## PROGRESS IN THE GLOBAL EFFORT TO ROLL BACK MALARIA

KAMINI MENDIS

Roll Back Malaria Project, World Health Organization, Geneva, Switzerland

The Roll Back Malaria (RBM) partnership to reduce the global burden of malaria has now been in operation for nearly 3 years. Established on the principles of tackling malaria as a health and development problems and using malaria to strengthen the health systems of malaria endemic countries, the RBM partnership has increased awareness of malaria as a major cause of poverty and underdevelopment, and inspired action against it at global, regional and country levels. RBM is a global effort with a major focus on Africa, which suffers the greatest burden of malaria. The technical strategies of RBM are 1) the provision of early and effective treatment against malaria, 2) prevention against malaria using insecticide-treated nets and other mosquito control methods, 3) reducing the impact of malaria in pregnancy and 4) preparedness to tackle malaria epidemics. Technical issues such as resistance to antimalarial drugs are being handled at a global and regional level through better surveillance, and arrangements to foster new drug development. The RBM movement is underpinned by a strong reseach and development (R&D) component-working with partners at all levels facilitating R&D on implementation and operational research to improve the delivery of existing tools, but also advocating for longer-term investments for new tools such as vaccines and new drugs.

Countries in sub-Saharan now have technically sound RBM strategic plans embodying RBM principles. These strategic plans are technically sound and use evidence based approaches and are integrated into the national health sector development plans. RBM is being used by countries to access financial resources through sector-wide approaches and poverty reduction strategies such as the Heavily Indebted Poor Country (HIPC) initiatives. Resource short-falls for 12 countries in Africa which are now ready to implement RBM are in the range of 0.5 billion US Dollars and global efforts to bridge these gaps are under way. In other regions of the world, the Mekong countries in Asia, and the Amazon region in South America are working as alliances to roll back malaria.

## MIM/TDR TASK FORCE ON MALARIA RESEARCH CAPABILITY STRENGTHENING IN AFRICA

FABIO ZICKER

Research Capability Strengthening, UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, Geneva, Switzerland

The medical and social-economic burden of malaria remains a major public health challenge in Africa. The reduced population access to effective treatment, rapid spreading of antimalarial drug resistance and vector resistance to insecticides have limited the impact of interventions on malaria morbidity and mortality. The development of a sustainable malaria research community in Africa is considered a priority to develop and improve public health interventions and policies.

The Multilateral Initiative on Malaria (MIM) is an alliance of organizations and individuals working together to maximize the impact of scientific research against malaria in Africa (http://mim.nih.gov) It aims to raise international public awareness of the problem of malaria, promote global research collaboration and co-ordination to improve malaria research capacity in Africa. MIM was launched at the conference on “Malaria in Africa: Challenges and Opportunities for Co-operation” held in Dakar (January 1997). Scientists and decision makers from Africa and from several international institutions met with funding organizations and identified scientific questions to be answered in order to address the problem of malaria in Africa. A multilateral funding mechanism, including Japan, was set up by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) to address these research questions and promote research capacity. The MIM/TDR Task Force is supporting a total of 23 multi-country partnership projects between African scientists (engaged in

basic and/or applied science) involving 24 African countries and investigators in developed countries with focus on strengthening the African research groups to *develop effective control tools malaria and improve relevant health policy strategies*. The overall aim is to generate new knowledge, develop products and intervention processes relevant to the understanding of the occurrence, distribution, morbidity and control of malaria in Africa.

The Task Force addresses the following priority areas identified at the Dakar meeting: antimalarial drug policy and chemotherapy, epidemiology, pathogenesis and immunology, entomology and vector studies, health systems research including social science, and natural products and drug development. The partnerships has provided opportunity for studying specific aspects of malaria in multiple sites, establishment of regional networks, and training of PhD and MSc students.

The projects under way will contribute to the development of:

- Strategies for mapping, retarding and reversing antimalarial drug resistance
- New methodology for improving malaria home management
- Surveillance network on malaria mortality/morbidity to support control initiatives
- Network on vector biology, insecticide resistance and vector population genetics
- Identify potential antimalarial compounds from natural products
- Studies on the relationships between parasite diversity, immune response, resistance, transmission, infection and disease patterns
- Better understanding of parasite-vector-host factors involved in severe disease.

Special lecture

## I THE FUTURE OF GLOBAL VACCINE RESEARCH

YOSHIFUMI TAKEDA

Faculty of Human Life Sciences, Jissen Women's Univewsity

Vaccines are designed to produce long-term protective immune responses against pathogens and are the most effective medical interventions used to prevent human morbidity and mortality associated with infections diseases. The eradication of the dreaded viral infection caused by human poxvirus is the best success story of how the efficient use of an effective vaccine can eliminate a disease, globally. Dramatic reductions in the incidence of numerous other childhood diseases, such as poliomyelitis, measles and rubella also show the success of efficient vaccines. To date most of the diseases where vaccines have been effective are diseases in which no vectors are involved. A vaccine strategy in diseases in which a vector is involved appears to be considerably more complicated and an outstanding exmaple is malaria. In contrast, enteric diseases like cholera and shigellosis, an effective vaccine should be easier to implement since a vector is not involved in causing the disease and since there are no known animal reservoirs of the causative agent. However, the exact type and quality of immunity afforded by natural enteric infections is still not clearly understood in these enteric infections. Rapid advances in immunology, molecular biology and peptide biochmistry have fostered the development of new approaches in the development of vaccines including the use of peptide vaccines and genetic vaccines. The future to conquer enteric diseases and other infectious diseases will largely depend on how successful the host of new enteric vaccines will be that are being developed using there new approaches. The WHO Vaccine Trial Registry documents an expanding research capacity with an average of 3.9 new studies per year during 1987-1933 rising to 10.7 per year during 1994-2000 with nearly 80% of these studies being conducted in developing countries. This is a good trend for the future and it is believed that over the next few years effective vaccines will be in place for some of most dreadful infectious diseases.

## II ALTERED IMMUNE RESPONSES IN MICE WITH CONCOMINTANT *SCHISTOSOMA MANSONI* AND *PLASMODIUM CHABAUDI* INFECTION

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Mixed patasitic infections are common in human populations living in malaria-endemic areas. However, little is known how concurrent infections affect the immunity and/or pathogenesis to each other. Protection and elimination of blood-stage *Plasmodium chabaudi chabaudi* As in resistant mice are characterized by a sequential activation of $CD4^+$ Th-1 and Th2 cells. The patent egg-laying stage of the murine model of *Schistosoma mansoni* is associated with a strong Th2 response to *Schistosoma* as well as unrelated antigens. The present study was performed to examine if *S. mansoni*-induced Th2 type of responses would prevent or inhibit the Th1 response required for the early clearance of *P. chabaudi*, thus giving rise to a more severe disease and/or mortality. Our data show that malaria parasitemias were significantly higher in mice infected with malaria at the time point of a patent egg-laying *S. mansoni* infection. *In vitro* stimulation with experiments using spleen cells showed that TNF-$\alpha$ production was sighnificantly impaired during the first week of the malaria infection in mice with concurrent *S. mansoni*/*P. chabaudi* infections. In contrast, the *P. chabaudi* induced Th1 type as measured by IFN-$\gamma$ production, was not affected by the *S. mansoni* infection. Taken together our date suggest that schistosoma and malaria infections profoundly affect each other, finding which might have implications for the development of future vaccines.

## S－I－1) CHOLERA

SHINJI YAMASAKI

Laboratory of International Prevention of Epidemics, Department of Veterinary Sciences, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University

Cholera is still a fatal disease in developing countries. Among 138 serogroups of *Vibrio cholerae* only O1 serogroup was thought to have potential to cause epidemic and pandemic of cholera. In 1992, however, a non-O1 *V. Cholerae* was involved in large outbreaks of cholera in the southern part of Inida. After preliminary serotyping studies, this strain was designated as *V. cholerae* O139 synonym Bengal. The O139 strain rapidly spread to neibouring countries such as Bangladesh, Nepal, Thailand and China, indicating that this strain had the potential to cause a cholera pandemic.

Since that time we have initiated several collaborative studies with National Institute of Cholera and Enteric Diseases, Calcutta on the molecular epidemiology and characterization of *Vibrio cholerae* strains isolated from diarrheal patients admitted to ID hospital. Through these studies, we have demonstrated followings, (1) predominant serofroup of *V. cholerae* isolated from the patients through the active surveillance between 1992 to 2000 was O1 serogroup and no O1 serogroup was isolated between January to July in 1993 when O139 was predominantly isolated during this period; (2) O1 *V. cholerae* isolated after O139 epidemic was not similar to those isolated before O139 epidemics; (3) the 20 kb region responsible for O1 antigen biosynthesis was replaced by a 36 kb fragment carrying the O139 specific genes in *V. cholerae* O139 and the 36 kb gene was most homologous to that in O22 serogroup; (4) the O1 and O139 specific genes were identified and a multiplex PCR, which can simultaneously amplify the ctxA, O1 specific and O139 specific genes was developed; (5) during the course of surveillance non-O1, non-O139 *V. cholerae* was also isolated from patients with severe diarrhea particularly during certain periods and these strain did not carry any known virulence factors normally present in toxigenic *V. cholerae O1*; (6) antibiotic resistant strains including multidrug resistant and resistance to new quinolones were observed and (7) antibiograms of O1, O139 and non-O1, non-O139 serogroups differed between each other during any given period.

## S－I－2) PANDEMIC SPREAD OF VIBRIO PARAHAEMOLYTICUS INFECTION: AN ENTERIC INFECTION SPREAD FROM A TROPICAL ZONE IN ASIA TO THE WORLD?

MITSUAKI NISHIBUCHI

Center for Southeast Asian Studies, Kyoto University

High incidence of *Vibrio parahaemolyticus* infection has been reported in the tropical and surrounding areas in Asia since 1996. The research done by our network revealed that it was due to the pandemic spread of *V. parahaemolyticus* infection. We first investigated the reason for a sharp increase in the number of *V. parahaemolyticus* infection in Calcutta in February 1996, and found that the increase was due to the infections by a newly emerged *V. parahaemolyticus*. The new type of *V. parahaemolyticus* was characterized by O3:K6 serovar, possession of the *tdh* gene, lack of the *trh* gene, and unique profile of arbitrarily primed PCR. The strains belonging to the same type were detected among those isolated from international travelers coming from three Southeast Asian countries to Japan. Subsequently, we conducted a multinational collaborative study and revealed that infection by the new type of *V. parahaemolyticus* occurred very frequently in Bangladesh, Thailand, Laos, Taiwan, Korea, Japan, and United States. We developed a new PCR method targeting the bases of the *toxRS* operon unique to the new type of *V. parahaemolyticus* so that it can be detected by this simple PCR method. Examination of many clinical stains by this PCR (named as GS-PCR for group-specific PCR) allowed us to find that the strains belonging to the new type of *V. parahaemolyticus* but exhibiting O4:K68 and O1:KUT serobars emerged in 1998. We named the new type of *V. parahaemolyticus*, including these serovars, a pandemic clone. DNA fingerprinting analyses by pulsed-field gel electrophoresis and ribotyping illustrated clonality of and subtle difference among the pandemic strains. Next, we were able to isolate an O3: K6

strain belonging to the pandemic clone from bloody clam in Hat Yai City in southern Thailand. A total of 317 strains of *V. parahaemolyticus* were isolated from diarrhea patients during one-year survey in two hospitals in Hat Yai City in 1999. GS-PCR-positive strains accounted for 75.4% of the isolated and 80% of the GS-PCR-positive strains belonged to O3:K6 serovar. The results demonstrated strong relationship between clinical and environmental O3:K6 strains. In addition, GS-PCR-positive strains belonging to three new serovars were detected, indicating emergence of the serovariants in this environment. The new serovariants of the pandemic strains were also detected among the international travelers from Thailand and Viet Nam. We also are examining about 600 clinical strains isolated in Nhatrang, Viet Nam to further expand our study on the epidemiology of the pandemic strains in Southeast Asian countries.

## S−I−3) *SALMONELLA*

HIDEO HAYASHI

Department of Infection Biology. Institute of Basic Medical Sciences, University of Tsukuba

*Salmonella* causes two major types of infectious diseases, enteric fever and food poisoning. The enteric fever is common in tropical countries from where travelers got infected and bring into the developed countries. According to the census of NIID, Japan, the reported cases of enteric fever in Japan was 148, during the period from April '99 to November '00. Among the cases, 108 (73%) were imported from tropical countries and the infected patients were almost men at their age of 20's. On the contrarily, salmonellar food poisoning which causes limited enteritis in man has been the most common in developed countries. Salmonella has been the top ranked food poisoning causative agent for many years in Japan. S. Typhimurium and S. Enreritidis have been the common agents but, in 1999, there happened a diffuse out break by S. Orenienburg that spreadded through dried squid-snack. We examined the specificity of the outbreak strains. The genomic pattern by PFGE was identical among the outbreak isolates but different from those isolated in Thailand and the isolate from the environment. The outbreak isolates had a couple of small plasmids and this was specific among the isolates from Ibaragi prefecture. The function of the plasmids are under investigation, but the reason why this train caused sudden out break has not been clarified.

Genus *Salmonella* contains more than 2,000 serotypes and ranges over variety of hosts from lower reptiles to higher primates. The virulent factors are also very diverese: it has almost all of bacterial virulence factors such as, adhesin, invasin, impedin aggressin, modulin and secretable enzymes. Those natures make it difficult to identify pathogenic strains to human with a simple and rapid method. The whole genome analysis was thought to be the most efficient method to solve those complex problems. The nine of the prototype strains have been subjected to the whole genome analysis but so far none of them is publicized as yet. The problems seem to lie in the variety or the flexbility of the genome structure and the complexity of regulatory mechanism of the gene expression. The five kinds of pathogenicity islands have been characterized but the relation to the serotypes or the distribution among the species are still indistinct. To prevent *Salmonella* infection, it needs a specific detection/identification method for human specific virulence factors.

We have been challenging to solve these problems by some different approaches: genetic analysis and identification of human specific virulent genes, detection of the specific gene by rapid PCR, identification of serotype specific genes by DNA chip and rapid PCR method, development of ELIZA for detection of enterotoxin in foods and clinical samples. With the application of gene subtraction method, we had been detecting human specific virulent genes and isolated seven of 2kb DNA fragments of which function remain to be identified. The developed methods mentioned above were briefly introduced.

This study was carried out by the cooperation with, H. Kurazono, Okayama University, S. Makino, Obihiro Veterinary College, S. Nakayama, NIID Japan, W. Chaicumpa, Mahidol University, P. Sudarmono, University of Indonesia, T. Kumao and W. Ba-Thein, University of Tsukuba.

## S－II－1) IMPORTANCE OF ACUTE RESPIRATORY INFECTIONS (ARI) IN TROPICAL AND DEVELOPING COUNTRIES

KAZUNORI OISHI, HIROYUKI YOSHIMINE, AND TSUYOSHI NAGATAKE

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Acute respiratory infections are common causes of morbidity and mortality in tropical and developing countries. A changing pattern of these diseases has, however, been recognized especially in Sub-Saharan Africa, where there is a high seroprevalence of HIV infection.

A hospital-based prospective study on a total 99 patients with community-acquired pneumonia (CAP) was carried out in Kampala, Uganda between 1996～1998. We evaluated the microbiological etiologies, clinical features and effectiveness of short-term parenteral ampicillin followed by oral amoxicillin for these patients according to HIV-status. We demonstrated a very high prevalence (74.8%) of HIV-1 infection. No significant difference was observed with respect to age, gender, prior antibiotic usage, symptoms, laboratory data or bacterial etiology between HIV-1-infected and HIV-uninfected CAP patients. Most of Streptococcus pneumoniae (19 strains) and Heamophilus influenzae (8 stains) isolated from HIV-1-infected patients were penicillin-resistant (94.7%) and b-lactamase producing (75.0%) stains, respectively. We, however, found a high percentage of good clinical response both in HIV-1-infected (81.3%) and HIV-uninfected (85.7%) among 39 patients with CAP due to bacterial pathogen. In addition, we documented a clinical feature of fifteen cases of recurrent bacterial pneumonia among 101 HIV-infected persons with CAP between 2000～2001. The mean CD4 levels of these patients were found to be 192/ml, and the mean interval between the first and the secound episode was 155 days.

Our present date suggest a clinical importance of bacterial CAP among HIV-infected persons and usefulness of short-term parenteral ampicillin therapy for these patients in HIV-endemic, developing countries.

## S－II－2) VIRAL ACUTE RESPIRATORY INFECTIONS IN TROPICAL COUNTRIES-FOCUS ON INFLUENZA VIRUS INFECTIONS-

SUZUKI HIROSHI

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Niigata University Graduate School of Medicine and Dental Sciences

Respiratory infections are the most common afflictions of humans, and mostly are caused by viruses. Children contract on average about half a dozen respiratory illnesses each year, and adults perhaps two or three. While many people consider influenza to be a minor disease, this may be due to be a minor disease, this may be due to confusion in the diagnosis. Symptoms of influenza resemble those of other infections and the term is often misapplied to a range of minor upper respiratory tract infections as a respiratory syndrome. More over, episodes of bacterial pneumonia, and worsening of underlying conditions, may not be recognized as being a consequence of prior influenza virus infections. Laboratory diagnosis is essential to verify the circulation of influenza viruses, and to generate reliable date on morbidity and mortality, especially in tropical or subtropical regions.

In most tropical subtropical regions, laboratory-confirmed influenza can occur throughout the year, with peaks of increased activity once or twice each year. When influenza may be not noticed because of a lower peak demand on health services. Furthermore, there were quite limited epidemilogical date of influenza infections in these regions due to lack of laboratory personnel and equipment.

Viral isolation is a basic and important technique in the field of virology despite the development of moderm techniques. One cell line can only culture a limiting numbers of these respiratory viruses, and has made it difficult to use the virus isolation as a routine method for laboratory diagnosis and epidemiological work of acute respiratory infections (ARI). In order to overcome this difficulty, we used the microplate method for virus isolation, which utilizes five cell lines and thus covers a wider range of respiratory viruses. Application of the method contributes to the routine diagnosis and helps define the epidemiology of viral ARI.

As influenza has consistently been associated with excess mortality and observed mainly in patients aged 65 or older, influenza vaccines are strongly recommended in countries for those persons who are at increased risk of complications. Hong Kong and Taiwan have influenza vaccination program for elderly people, but not other Southeast Asian countries. In more then 1 peak of influenza occurs each year, we need to study the appropriate timing of vaccination and selection of the vaccine formation.

## S－II－3) BURDEN OF *HAEMOPHILUS INFLUENZAE* TYPE B (HIB) DISEASES IN BANGLADESH

SAMIR K SAHA[1], M. HANIF[1], M. RUHULAMIN[1], K. MATSUMOTO[2], K. OISHI[2], T. NAGATAKE[2]

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The virtual elimination of *Haemophilus influenzae* type b (Hib) disease from industrialized countries by vaccine is a moderm public health triumph. However, the disease is still prevalent in the developing part of the world, where the vaccine is not available, and causes invasive diseases like pneumonia, meningitis etc. Hib diseases cause 12.9 million deaths per year in under five children and most of them are in the nonindustrialized countries. In the recent years, the problem is further aggravated due to the emergence of multidrug resistant Hib strains. Surveillance on burden of Hib diseases is focused on meningitis cases as the diagnosis is straight forward and can yield a very accurate estimates of over all burden of the disease.

In the study period, from 1994 to 2000, 338 invasive strains of *H. influenzae* were isolated from blood and CSF of pneumonia and meningitis cases respectively. *H. influenzae* was the most predominant (50%) among the isolates followed by *S. pneumoniae* (32%), and 97% of the all *H. influenzae* isolates were type b (Hib). A vast majority (91%) of Hib infections were found in first year and only few (2%) after 2nd year of life.

Over all 35%, 54% and 16% of the strains were resistant to ampicillin, cotrimoxazole and chloramphenicol respectively. Most (95%) of the ampicillin resistance was due to data-lactamase production, as detected by nitrocefin disk, and the MIC level these strains were>256.0 ug/ml. Cotrimoxazole resistance showed an increasing trend with peak (64%) in 1996 and then declined to the level of 33% in 2000. Resistance to ampicillin remained similar through out the study period (range 33% to 43%). The most notable finding was the emergence of resistance to chloramphenicol in 1996 (5%) and its increasing trend in the subsequent years, which reched to the level of 40% in 2000.

Meningitis caused by Hib resulted death in 34% and similar number were survived with sequel. Eighty four percent of the children with Hib diseases were from very poor families who cannot afford to buy the medicines and vaccines for treatment and prevention respectively.

These findings has paramount implications in child health as cotrimoxazole is recommended by WHO for pneumonia, at the community level, and ampicillin and chloramphenicol is the most available and widely used antibiotics in the hospitals of this country. Lastly, the predominance of Hib in causing invasive diseases and emergence of resistance among them strongly indicates the need of introduction of Hib vaccine in EPI program of Bangladesh.

## S－III－1)　RECENT DEVELOPMENTS IN HIV/AIDS RESEARCH

TAKASHI KITAMURA

Director Emeritus, Nattional Institute of Infectious Diseases/Toyama Institute of Health

"Panel on AIDS" of US-Japan Medical Science Program (CMSP) has been working with US Panel to combine the research activities and achievements on HIV/AIDS carried out in both United States and Japan. As the Japanese Panel chair, the speaker has been watching the developments of global HIV/AIDS pandemic and researches to fight against it. Here, as the first speaker of the session, I would like to present the recent developments in HIV/AIDS pandemic and researches in various aspects against it.

**1) Epidemiology**; AIDS was recognized in 1981 by US-CDC. By its excellent studies based on surveillance it could identify several key risk factors by March 1983, before the discovery of causing virus by Montagnier in May 1983. By the end of 2000, estimated 36 million people have been infected with HIV, more than 70% clustering in sub-Saharan Africa. Number of AIDS orphans, children lost both parents due to HIV, is increasing rapidly in many developing countries.

**2) Blood safety**; Safety of the blood supply has been ensured by the donor screening based on the detection of HIV antibody. There is "acute infection phase", a few weeks after HIV exposure, where relatively high level of viremia makes the donor highly infectious despite the "sero-negative" test results. To close such "window period", viral nucleic acid testing has been introduced in Japan to close the "window" by nearly 30%.

**3) Vaccine development**; Retroviruses have "latent infection period" as a part of regular replication cycle and, during this period, HIV-infected cells can escape the surveillance of immune mechanisms, resulting in the theoretical difficulty of vaccine development. Recently Prof. Kiyono's group identified "common mucosal immunity" among various mucosal compartments. Nasal immunization of animals could induce vaginal immunity against recombinant virus with HIV/SIV genes.

**4) Chemotherapy**; Retroviruses from an exceptionally "chemotherapy-prone" virus group. They have in their replication cycle several specific steps vulnerable to drug interventions. Successful developments of reverse transcriptase and protease inhibitors substantially improved the prognosis of HIV/AIDS patients, reducing the progression of HIV disease and death by AIDS. Mother-to-child tansmission are effectively prevented and occupational exposures can be managed by chemotherapeutic regimens.

**5) Transmission**; Transmission efficiency has been studied quantitatively by assay of viral RNA genome loads. Risk of being infected by single episode of heterosexual contact is estimated to be 0.05-0.15% (infection after 700-2000 contacts). Ulcerous or inflammatory lesions in the genital mucosa enhances the risk of HIV transmission. Genetic deletion of a co-receptor, CCR5, expression make the individual HIV-resistant.

**6) Prevention**; UNAIDS put the prevention of general STDs as the most effective preventive approach against HIV infection. Human trial of "topical vaginal microbicide" containing spermicidal contraceptive enhanced the HIV transmission by nearly 50%, possibly due to inflammatory changes in mucosa caused by unexpectedly frequent applications.

**7) Molecular epidemiolgy**; HIV can be identified with subtypes (clades) based on base sequences of individual genes. With the time after the introduction of multiple subtypes of HIV in circulation within a region, increasing variety of recombinants were identified. Now the circulating HIVs are described by CRF (circulating recombinant form) designation.

**8) Drug resistance**; Resistances to various antiretroviral drugs are idetified as the mutations in pol genes. Diagnostic tests to identify the infectious viral genomes and possible resistance mutations in the sequence are being developed, in response to the need of effective therpeutic design.

## S－III－2) RESEARCH AND DEVELOPMENT OF RECOMBINANT HIV VACCINE-PRE-CLINICAL TRIAL

HONDA M., NAKASONE T., MATSUO K., IZUMI Y., HAMANO T., OHSU T., TAKIZAWA M., SOMEYA K., KAIZU M., HARA T., KAWAHARA M., HORIBATA S., KENEKIYO M., HAMATAKE M., AMI Y., SAKAI K., SHINOHARA K., SAKAKI Y., KURATA T., YAMAZAKI S., AND YAMAMOTO N.

National Institute of Infectious Disease

In controlling the HIV infection, preventive HIV vaccine would be of great use, when it was developed efficaciously. Recent considerable progresses have shown that correlates of protective immunity may due to interaction of immunological, viral and genetic factors. Furthermore, high levels of the cellular and humoral immune reponses are probably responsible for controlling the viral loads that limit HIV infection and progression of diseases. *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) and a vaccinia viral strain that trageted in this HIV vaccine development are considered to be the appropriate HIV vaccine-vector that satisfy the requirements for HIV vaccine use for human, when it would induce high levels of HIV-specific immunity with maintaining BCG immunity against tuberculosis. The prime-boost vaccine regimen significantly protected all the animals from high levels of palasma viral load and $CD4^+$ cell loss: a decline in HIV1 set-point levels was more than hundred times and $CD4^+$ cell decline was also significantly reduced. This approach induced similar levels of immunity-specific for HIV-1 Gag in animals consecutively primed with rBCG-HIV/Gag (80 mg orally or 0.1 mg intradermally) and boosted with rDIs HIV/Gag. Since the vaccine regimen is revealed to be very safe, these studies suggest a strategy for enhanced induction of T-cell mediated protective immunity to HIV-1.

## S－III－3） TREATMENT OF AIDS

MASAKAZU HATANAKA

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AIDS, acquired immunodeficiency syndrome, is caused by infection of a retrovirus called HIV, human immunodeficiency virus. HIV encodes three enzymes, reverse transcriptase, protease and integrase, whose inhibition is the targets of AIDS treatment.

First, nucleoside analogs has been developed as inhibitors of reverse transcriptase followed by non-nucleosides. As non-nucleoside-inhibitors are less expensive compared with nucleoside analogs, developing countries may afford their use for the treatment of AIDS and prevention of mother-to-child infection of HIV. Although the onset of resistant strains of HIV towards reverse transcriptase is inevitable by long duration of treatment course, adequate use and change of drugs may lead long threapeutic effects. Protease inhibitors have been developed by mainly molecular designing and are quite effective against resistant strains of reverse transcriptase inhibitors and presently combination of three drugs against reverse transcriptase and protease is a major stream of treatment of AIDS called HAART (highly active anti-retroviral therapy). At the first time, we have developed an inhibitor of HIV integrase (S-1360), which is under a clinical trial in USA and is expected to be soon available for the AIDS patients. In developing countries of Africa and Asia, HIV infection is in the midst of its pandemics and is urgent to take action of its prevention and treatment, which is alerted as emergency by United Nations and WHO. Developed countries including Japan are now taking actions urgently towards this target in many extensive ways including medical and financial supports.

## S－IV－1) TRIALS AND PROSPECTS OF EPIDEMIOLOGICAL SURVEY FOR BABESIOSIS IN JAPAN AND MALARIA-ENDEMIC AREAS

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Babesiosis is tick-transmitted intraerythrocytic protozoan disease of animals and occasionally occurs in humans. Most cases of human babesiosis have been caused by *Babesia microti*, a species of rodents. Cases by *B. microti* have been reported nearly exclusively from the United States. In 1999, we confirmed the first human babesiosis case in Japan. It was demonstrated that the case had been infected by the blood transfusion from an asymptpmatic donor infected with *B. microti*-like parasites. The sequence of SSUrDNA of parasites isolated from the patient (Kobe srtain), however, was distinct from that of *B. microti* of the United States origin (Gray, GI and AJ strains) nor that of parasites isolated from wild rats in Otsu where *B. microti*-like parasites were first demonstrated in wild rats in Japan (Otsu strain), although they have the higest homology to one another. Antigenicities of Kobe strain, Otsu srtain and the strains of US origin are also different from one another.

In these circumstances, we examined antibody-titers against Kobe strain by IFAT for 995 sera in 2000 and 499 sera in 1981 of outpatients of hospitals in Hyogo under informed consent. Three sera2000 and one in 1981 showed to be positive at ≧x518 including two positive at ≧x4096. One serum postive at ≧x518 showed no cross reactivities against Otsu strain or *P. yoelii* like the sera of the first patient and the asymptomatic donor. By western blot analysis, it also detected the antigen of the same size (ca. 40 kD) as that for the sera of the first patient and the asymptomatic donor. Thus , it was suggested that at least one outpatient, the donor of the serum, had experienced the actual infection with Kobe strain. However, three sera that showed cross reactivities against Otsu strain and *P. yoelii* could be false-positive. Specificities of IFAT should be reconfirmed.

In the field survey of rats and ticks for *Babesia* infection in Hyogo in 2000-2001, it was showed by microscopic observation of smears or PCR that 5 of 9 rats were *B. microti*-positive in Rokko area, and all 5 were Otsu type. In Awaji island, 6 of 22 rats were *B. microti*-positive, and 4 were Kobe type and 1 was Otsu type (1 was not detemined). Morever, the probable SSUrDNA of *B. microti* was amplified from a salivary gland of a tick of *Ixodes ovatus* collected in Rokko. Its sequence has the highest homology with those of Kobe, Otsu and US type, but different from any of them. It was suggested that *I. ovatus* has some roles in transmission of *B. microti*.

In the field survey of rats for *Babesia* infection in Chiang Mai, Thailand in 2000, *Babesia*-like parasites were identified in 6 of 9 of *Bandicota indica*. The sequence analysis of the parasites showed that the parasites are more closely related to *B. canis* and *B. divergence* than *B. microti*. Although futher studies are necessary, the emergence of human babesiosis should be considered in malaria endemic areas.

## S－IV－2) NOVEL STRATEGIES FOR TICK CONTROL

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Ticks are ectoparasites found in almost all parts of the world. While mosquitoes trasmit most of the important human pathogens such as malaria and yellow fever, ticks surpass all other arthropods in the number of diseases they transmit to animals and humans. Damage resulting from direct tick feeding activities and the cost of controlling the pathogens they transmit to libestock accounts for the lagest source of economic losses in the livestock industry. Currently the only widely available practical method to control ticks is the use of chemical acaricides. This approach is associated with serious limitations such as environmental and food chain contamination by acaricides. These limitations

and the growing community awareness regarding the harmful effects of acaricides have stimulated research into alternative tick control methods. Among the several alternative tick control methods that have been considered, immunological protection of hosts against tick infestation is practical and sustainable. Findings from a series of studies by an Australian group leading to commercialization of a first ever anti-arthropod vaccine, e. g., TickGARD and Gavac, provided practical evidence on the use of an immunological approach to control ticks. However the success of this approach is dependent upon identification of key tick molecules that are involved in the mediation of key physiological roles for use as candidate vaccine antigens. From this stadpoint, we made here the brief introduction about our recent achievements on the identification and characterization of several candidate molecules, namely phenoloxidse, serine-protease, peroxiredoxin and troponin, from several tick species.

## S－IV－3) SIMIAN MALARIA PARASITES AS ZOONOTIC PATHOGENS

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The parasites of genus *Plasmodium* have a wide range of vertebrate hosts. Although they are generally known to have strict host specificity, some simian malaria parasites are infective to human beings, causing zoonotic infectious diseases. Here we describe simian malaria parasites zoonotic pathogens.

To date, about 25 spices of simian maralia parasites have been reported. These occur in New World monkeys, Old World monkeys, gibbons, great apes, and lemurs. *Plasmodium* of the primate hosts are classified into five groups according to Giemsa-stained feature of stages found in the peripheral blood and the periodicity of their erythrocytic schizogony: falciparum type, vivax type, maraliae type, ovale type and other type. At least six species of simian malaria parasites (*P. brazilianum*, *P. cynomolgi*, *P. inui*, *P. knowlesi*, *P. schwetzi* and *P. simium*) are known to infect humans either naturally or experimentally including accidental laboratory infections.

Generally, the clinical course of infection with simian malaria parasites in humans is mild with low parasitemia, and chemotherapy, where necessary, has been effective. Although human malaria infections acquired from non-human primates are rare, an accurate diagnosis should be made in any suspected cases. Diagnosis is based on the detection of parasites in stained thin blood smears but these must be interpreted with great caution because simian malaria parasites are virtually indistinguishable from the erythrocytic stages of human malaria parasites.

Some epidemiological studies on simian malaria have pointed out the possible role of malaria infected monkeys as natural reservoirs for infection in humans. *P. brazilianum* is widely distributed in several species of monkeys in Central and South America. This malariae type species is morphologically and molecularly similar to human parasite *P. malariae*, thus they are virtually identical and should probably be considered to be a single malaria species. In fact, serological studies in the Amazon Basin have shown the prevalence for anti-*P. brazilianum* /*P. malariae* antibodies to be high both in monleys and in local Indian tribes, suggesting that this from of malaria is authentic zoonosis in that region. These findings indicate the possibility that monkeys living in the rainforest of Amazon area are natural reservoirs for both simian and human malaria.

## S－IV－4) LEISHIMANIASIS AS A ZOONOSIS: DNA DIAGNOSIS AND MOLECULAR EPIDEMILOGY BASED ON PCR AMPLIFICATION AND CHARACTERISTICS OF THE LEISHMANIA MINI-EXON GENE

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Leishmaniasis is typical vector-borne and zoonotic disease. The disease is transmitted by the sandfly and more than 100 vertebrates are known as resevoir hosts. To control leishmaniasis most effectively, an accurate diagnosis of patients well as epidemiological studies on reservoir animals and sandfly vectors are required. For DNA diagnosis of leishmaniasis, we have developed a PCR method based on amplification of the *Leishmania* mini-exon gene. The mini-exon gene is tandem repeated as many as 100-200 copies on the parasite genome and the spacer region is defferent in length and in sequence among different *Leishmania* species. We evaluated the usefulness of this PCR method in two endemic countries, China and Ecuador.

In Xinjiang-Uigur Autonomous Region in China, there have been sporadic cases of visceral leishmaniasis. We obtained positive PCR products corresponding to *L. donovani* complex in bone marrow or buffy coat samples in 11 out of 13 serologically positive kala-azar patient. However, none of buffy coat samples from nine patients with antimony were all PCR-negative. DNA samples from buffy coats of 17 domestic in the endemic area were also PCR-negative. In addition, we identified parasite isolates from great gerbils (*Rhombomys opimus*) and sand flies (*Rhlebotomus mongolensis* and *P. andrejevi*) as *L. turanica* or *L. gerbilli* by DNA sequencing of the PCR-amplified mini-exon genes.

In Ecuador, amplification of the mini-exon gene specific to *L. braziliensis* complex was detected in the skin biopsy sampls from patients with cutaneous leishmaniasis, indicating the usefulness of PCR amplification of the gene for diagnosis of cutaneous leishmaniasis. Chromosomal locations of the mini-exon gene were different among *L. panamensis* isolates, futher indicating the DNA karyotype variation among parasites in the endemic region. Furthermore, DNA sequencing of amplified mini-exon genes of isolates from a sloth (*Choloepus hoffmanni*), a squirrel (*Sciurus vulgaris*) and sand flies (*Lutzomyia hartomani*) revealed the presence of *Endotrypanum monterogeii* and *E. schaudinni* in Ecuador. Since some of these isolates were reported as *L. equatorensis* in the previous paper, it is impottant for the differentiation between *Leishmania* and *Endotrypanum* for epidemiological studies on leishmaniasis.

## S－V－1) PROGRESS OF GLOBAL POLIO ERADICATION PROGRAMME

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The global polio eradication programme has launched by world health oraganization in 1988. The main strategy is (i) detecting patients with acute flaccid paralysis (AFP) and (ii) confirming real polio cases both by clinical and virological examination. If the number of AFP patients due to wild type-polioviruses becomes zero and if the situation is maintained for a certain period of time (～3 years), the programme is accomplished. In practice, the programme consists of extensive vaccination and highy effective surveillance. Since the key element is oral attenuated vaccine, this programme is considered to be, in other words, an experiment to replace wild polioviruses with vaccine strains in global level.

In the American Region, the programme was accomplished in 1991 and no AFP patients due to wild polioviruses have been reported in spite of extensive vaccination and surveillance. In our Western Pacific Region, the last polio case was found in Cambodia in March 1997, and since then no genuine polio cases by wild polioviruses have been reported. The certification committee declared in October 2000 that transmission of wild polioviruses has completely interrupted and now the region is wild polio-free. In the European region, the last polio case is considered to be the one in Turkey in 1998.

The initial target of the WHO programme was 2000. Although the accomplishments are delayed than expected,

the programme is now at the very final stage. However at the very end, the following problems became clear; (i) the areas where wild polio viruses still exist are the areas with serious internal war and international collaborative efforts are generally difficult to access (Afghanistan, Somalia, Angola, etc…), (ii) polio-free declaration in the regional level sometimes induce negative effects for the maintaining the high level of vaccination and sustaining good surveillance. The latter is particularly important because in some areas where wild polioviruses are no more detected, vaccine derived poliovirus caused poliomyelitis outbreak in low or non-immunized population. The eradication of poliomyelitis does not automatically mean the abolishment of vaccination. It is the time to consider how we can stop polio vaccination (polio end game).

## S－V－2) INTERNATIONAL COOPERATION IN THE AREA OF TUBERCULOSIS CONTROL

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Global burden of Tuberculosis and DOTS:

Tuberculosis is now a global burden as one third of the global population has been infected by *M.tuberculosis*, causing 8 million new cases and 2 million deaths every year, WHO has developde a global strategy called DOTS through the analysis of the success stories in the world. DOTS consists of the following 5 components; government commitment, microscopic examination for diagnosis to the symptomatics, safe and regular supply of TB drugs, direcr observation of medication with short course regimen, routine monitoring with recording and reporting system. DOTS has yet covered only half the global population and one third of the estimated number of patients, The international cooperation must be therefore strengthened to accelerate global expansion of DOTS.

International Cooperation for TB Control by Japan:

Japan has a long history of international cooperation in the area of TB control since early 1960s both through the government and NGOs. International training courses held at research Institute of Tuberculosis since 1963 have had a global reputation wit more than 1500 participants from 80 contries. New challenges are; donation of drugs which are needed for DOTS expansion, recruit of the local staff for the project, or support for the nation-wide expansion of the program. Flexible approaches are needed according to the needs and effects. Coordination with other and international agencies such as WHO, or NGOs should be actively made, and global commitment both technical and political need to be strengthened.

Support System and Base Establishment inside Japan:

The base capacity inside Japan should be further strengthened, for manpower development for international works, overseas project support, or collaborative research.

## S－V－3) MALARIA CONTROL PROGRAM IN LAO PDR SUPPORTED BY JAPANESE GRANT AID FOR CHILD HEALTH

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In Lao PDR, Malaria control program using Impregnated Bed Net (IBN) stated from 1998 in nation wide according to Roll Back Malaria Initiative. Project in three central provinces was supported by Japanese Grant Aid for Child Health under the supervision from CMPE, Ministry of Health and JICA expert. Total of 40000 bed nets and Insecticide (Deltamethrin) were donated to CMPE and three provincial health offices in the beginning of 1999. Until end of 2000, it was reported that 81% of bed bet was delivered to the communities. Commune (Sub-district consisted

by 5-10 villages) was applied as unit to deliver bed net cost-effectively. One village was selected to hold Training of Trainer (TOT) in each commune to expand to other villages by district malaria staffs, village leaders or health workers without support from central level. Total expense to deliver the bed net using these systems was approximately only US $10000.

Total number of malaria patients and slide positive rate of malaria (SPR) in all public health facilities decreased markedly in 2000. In the pilot district; Bourapha district in Khammoaune province, it was suspected that IBN program was well operated according to the analysis the report from district health office. The report showed 100% coverage to villages and 3.81 person per IBN as mean coverage in the community. After operation IBN program, KAP study was carried out to evaluate the program in all communes in pilot district. High percentage of household where bed net wasn't set was presented in the remote area. Moreover, low malaria knowledge was confirmed among inhabitants who live in household without bed net. It was suggested that strengthening of IEC activities is necessary in especially remote area and poor families. Thus, the new approach to the inhabitants was applied to sustain the project. To strengthen the community participation, group discussion and mass diagnosis using rapid diagnosis method were conducted before ordinary health education using video or poster. The efficacy of these trials will be evaluated in beginning of 2002.

It is expected that improvement of cost recovering system and application of social marketing system support to sustain the project with strengthening IEC activities.

## S－V－4) LEISHMANIASIS: ITS RECENT TOPICS AND RESEARCH ACTVITIES IN ECUADOR

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Leishmaniasis is one of the eight target diseases of the Improved Control of TDR diseases (WHO, 2000). The diseases are endemic in 88 countries on the five continents, with a total of 35 million people at risk, and the diseases afflict at least 12 million people worldwide; the numbers are however probably underestimated; figures of 2 million new cases per year, including 1.5 million cases of cutaneous leishmaniasis (CL) and 500,000 of visceral leishmanisis (VL) are likely (Desjeux, 2001). In this symposium, the following points are mentioned as recent topics in leishmaniasis research. *1) Epidemiological changes due to* Leishmania/*HIV co-infection*. Such cases are increasing yearly and the co-infection is prevalent in 33 countries, including non-endemic areas, such as Germany, Switzerland, United Kingdom, Belgium, Denmark and etc. In the co-infection areas, transmission cycles are also changing from zoonotic endemics to anthroponotic epidemics; person to person direct trasnsmissions by syringe in drug users are found, especially in south-western Eurpe. *2) Vaccine trials*. Recent vaccine trials performed for CL in Iran and for VL in Sudan, and for CL in Brazil, Ecuador and Colombia were briefly reviewed, including immunotherapy done in Venezuela. *3) Chemotherapy for leishmaniasis*. Available therapy for the disease was reviewed and special emphasis was given to the trials with miltefosine as oral drug for VL used especially in India. Besides, leishmaniasis research project carried out during about 18 years from 1982 to 2001 was retrospectively evaluated, mainly emphasizing on the following points: 1) characterization and determination of Leishmania spp., vector sandflies, and reservoirs at each endemic areas of Ecuador; 2) therapy trials using paromomycin ointment and Glucantime lotions as local treatment and Mephaquin and artesunate as oral treatment; 3) detection of epidemiological characteristics of each area and etc.; 4) development of material collections in the field condition for molecular diagnostic tools.

## S－V－5) CURRENT ACTIVITIES OF REGIONAL NETWORK ON SCHISTOSOMIASIS CONTROL IN ASIA AND ROLES OF THE JAPANESE RESEARCHERS IN THE NETWORK

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Asian schistosomiasis, schistosomiasis japonica, is endemic in east-Asian countries especially in China and the Philippines. In comparison with other human schistosomiasis, the disease control is difficult because of the presence of reservoir hosts and also environmental factors. Under the support of WHO, a regional network for controlling Asian schistosomiasis (RNAS) was established as international cooperation activities including the two endemic countries as well as other countries such as Japan and Australia, in which research on schistosomiasis has been accumulated. Endemic situation is different between China and the Philippines, therefore, control strategies are different between the two countries. In the previous joint RNAS meetings, methods for epidemiological evaluation, developing new diagnostic tools, and vaccine development were the common interest. Japanese researchers are requested to join the cooperative activities in such fields.

Endemic situation of schistosomiasis japonica in both two countries at a turning point, because WHO gave relatively intense supports for the disease control in the past decades. Situation has been much improved, however, support by WHO was already over by the end of 2000. In China, the number of endemic Prefectures and the number of advanced cases showed significant reduction, and better-organized countermeasures are needed. For example, selected chemotherapy is the choice in many places in China, however, such program management is now not easy because of financial problem. Reduced endemicity means difficult step for disease eradication and needs much more budget and human resources. The same situation is also in the Philippines. Along with social development, endemicity is deeply affected by some artificial conditions. The three-gauge dome in China is the case, Epidemiological suevey in Human, Hubei, Sichuan and Jiangxi Provinces are underway.

Cost-effective countermeasure is the most important subject this region. Technical and scientific assistance from Japan will be good contribution. In line with such situation, Japanese researchers are joining partnership for vaccine development, and for developing new epidemilogical evaluation. RNAS activity has just started, and should be expanded though the partnership in east-Asian countries. This is, in turn, a good opportunity for Japanese reseachers, since schistosomiasis is still attractive field of research.

## A－1) CHANGING PATTERNS OF ANTIMALARIAL DRUG RESISTANCE IN *PLASMODIUM FALCIPARUM* MIRROR THE CHANGE OF THE NATIONAL MALARIA TREATMENT POLICY IN MALAWI

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In Malawi chloroquine was replaced by sulfadoxine/ pyrimethamine (SP) in 1993 because of increasing chloroquine failures of *Plasmodium falciparum* cases. Seven years after the change in malaria treatment policy, we evaluated the sensitivity of *P. falciparum* to different antimalarial drugs both *in vivo* and *in vitro* in asymptomatic children and adults in Salima District, Malawi.

A total of 504 individuals were examined for parasitaemias. Of them, 78% were infected with malaria. Monoinfections of *P. falciparum* and *P. malariae* accounted for 97% and 1% of the positives respectively, while 2% had mixed infections. Children <6 years were mostly infected (93%) and had high parasite rate and spleen rate.

Of 173 subjects selected for *in vivo* trials, 61 were treated with chloroquine, 46 with SP, and 66 with Malarone (atovaquone/proguanil) and were followed for 28 days. The *in vivo* sensitivity response of the three drugs tested was not significantly different (78-88%, $p>0.05$), however, Malarone group showed a significantly higher proportion of RIII/ RII resistance than chloroquine and SP groups (9% vs. 2%, $p<0.02$).

The *in vitro* sensitivity of 104 *P. falciparum* isolates, to chloroquine, SP, pyrimethamine, amodiaquine, quinine, and mefloquine was also assessed using WHO test kits. Ninety eight percent of the parasites failed to mature at the lowest concentration of chloroquine ($0.2\mu mol/l$ BMM) precluding $EC_{50}$ and $EC_{90}$ estimates. Although 44% of the isolates showed schizont maturation at the highest concentration of pyrimethamine, 83% were inhibited at the cut-off concentration of SP combination.

Our study in Malawi shows the recovery of chloroquine sensitivity by comparing both *in vivo* and *in vitro* tests of the same *P. falciparum* isolates. The combination of sulfadoxine and pyrimethamine was effective in the presence of pyrimethamine-resistance isolates, indicating the advantage of drug combinations as opposed to single drug therapy in clinical practice. Thus, if chloroquine is removed for a sufficient period of time, it might be effectively used once again in combination with other drugs to treat malaria in Sub-Saharan Africa.

## A－2) ANALYSIS OF *PFMDR1* GENE IN MEFLOQUINE RESISTANT *PLASMODIUM FALCIPARUM*

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Objective: Drug resistance is a major problem for the treatment of malaria. Therefore new antimalarials are necessary to combat resistant parasites. Mefloquine is an important drug, but resistant parasite are also appeared and now increasing. Our objective was to analyze the mechanism of mefloquine-resistant of *Plasmodium falciparum* parasite to develop new drugs.

Methods: A mefloquine-resistant strain *Plasmodium falciparum* 523a (isolated from mefloquine-resistant patient) was continuously cultured under progressively increased mefloquine pressure. After two years, we got resistant parasite (designated as 523a R strain) and now can able to grow at $2.5\times10^{-7}$ M mefloquine concentration. The original strain was cultured without drug under the same condition

considered as control (523a S strain). The sensitivity to other antimalarials against these strains (523a S and 523a R) have also been performed. The amino acid residues at 86, 1034, 1042 and 1246 positions of *pfmdr1* gene in chromosome 5 of *P. falciparum* (523a S and 523a R) were analyzed using Dye Terminator method followed by particular domain amplified by PCR and compared with previously reported amino acid residues which thought to involved mefloquine resistance. The size of chromosome 5 has been measured. We performed cloning from resistant strain and determined the sensitivity to other antimalarials against clone/24.

Results and Discussion: It is found that, there is no difference in amino acid sequence between mefloquine sensitive (523a S) and mefloquine resistant (523a R) strain, although resistant strain showed about 39 times higher $IC_{50}$ value than sensitive strain. Moreover, 523a R strain showed decreased sensitivity to artemisinin and halofantrine and increased sensitivity to chloroquine. The overexpression of *pfmdr1* mRNA has been observed and it is about 7.2 times higher than sensitive strain. Our Pulsed Field Gel Electrophoresis result revealed chromosome 5 contained a mixed population of sensitive and resistant parasites. To get more resistant parasites we performed cloning. Clone/24, the highest resistant clone showed similar results as 523a R strain to other antimalarials, increased sensitivity to chloroquine and decreased sensitivity to artemisininin and halofantrine.

Our results may explain amino acid sequence should not related to drug resistance and sequence polymorphism of *pfmdr1* gene. As overexpression of gene *pfmdr1* found about 7.2 times higher than sensitive strain, it is likely that overexpression of *pfmdr1* gene may be associated in mefloquine resistance mechanism. Now we are measuring the size of chromosome 5, overexpression of mRNA and gene analysis of *pfmdr1* against clone/24.

## A－3) RECOVERY OF CHLOROQUINE SENSITIVITY AND LOW PREVALENCE OF K76T IN *PFCRT* AFTER THE CHANGE OF MALARIA TREATMENT POLICY IN MALAWI

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In 1993 Malawi officially withdrew chloroquine as the first-line drug for the treatment of uncomplicated cases of malaria due to an increasingly high rate (47%) of resistance. During field surveys conducted in 1998 and 2000 we investigated *in vitro* chloroquine resistance (CQR) and later assayed two genetic polymorphisms (K76T in *pfcrt* and N86 Y in *pfmdr1*) suggested to contribute to CQR in obtained *Plasmodium falciparum* isolates. Among 96 isolates examined, only 9% showed CQR while 7% and 48% carried the K76T and N86Y mutations respectively. In contrast, the prevalence of K76T in other African countries with ongoing chloroquine use was 41-83%. Thus, our findings suggest that the reduced drug pressure accompanying the change in national drug policy in Malawi has resulted in a significantly lower prevalence of the K76T mutation and consequently a substantial recovery of chloroquine sensitivity.

## A－4) DRUG RESISTANT MALARIA IN MINDANAO, THE PHILIPPINES-APPLICATION OF ANAEROPACK® MALARIA CULTURE SYSTEM TO *IN VITRO* DRUG SUSCEPTIBILITY TEST OF *PLASMODIUM FALCIPARUM* IN THE FIELD.

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Since chloroquine-resisant *Plasmodium falciparum* was reported in 1960's, drug-resistance or decreased susceptibility for drugs of malaria parasites have expanded their distribution. Therefore, it is important to carry out the drug susceptibility in the field to obtain the epidemiological information against the drug-resistant malaria parasites in detail. However, it is different to isolate the fresh parasite or to examine the drug susceptibility for antimalarial drugs in the field because of the limited experimental conditions needed for the cultivation of the *P. falciparum in vitro*. In this report, drug susceptibility of *P. falciparum* isolates was determined using AnaeroPack® malaria culture system with portable thermostat in the field of Mindanao Island, the Philippines. Blood samples were obtained from 4 symptomatic Filipino patients with *P. falciparum* who admitted to the Davao regional hospital, in Tagum City. The aim of the study was clearly explained to all the patients and informed consent was obtained from them. Erythrocytes were resuspended in RPMI 1640 medium supplemented with 10% human serum. The culture plates including the blood samples were incubated at 37℃ with portable thermostat. The 4 isolates were examined for the chloroquine-susceptibility by adding the drug to the cultured samples at serially diluted concentrations. After 36 hr incubation, 2 isolates grew from ring form to schizonts. Then, a 100% inhibitory concentration ($IC_{100}$) of one isolate was observed to be 1.28 $\mu$M and that of the other isolate was 0.64 $\mu$M. Two other Isolates of 4 grew to trophozoite as late as after 48 hours' incubation and were also thought of as isolates of a certain degree of resistance. AnaeroPark® malaria culture system with portable thermostat was proved to be powerful and useful to conduct the drug susceptibility test in the field condition.

## A－5) AN EPIDEMIOLOGICAL STUDY ON MALARIA IN THE DISPLACED POPULATIONS IN VIET NAM

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Malaria incidence is determined by a variety of factors, particularly the abundance of anopheline mosquitoes, the presence of malaria parasites, and human behavior. Re-emergence of malaria is thus often associated with socio-economic development and expanding infrastructure, population growth and urbanization. We report an example of malaria epidemics in the displaced populations in Viet Nam.

Study area was a newly colonized village called "Ben Tre-A New Economic Area" in Binh Phuoc province, which was located about a 100km to the north of Ho Chi Minh City and quite near to Cambodia border. People had moved from the area called "Ben Tre" near the mouse of Mekong river and settled there. Seventy families including 203 people were residing in the area and just started to cultivate the land growing rubber trees, nuts, pepper and rice for their support. Access to the village was not easy by walking but only feasible by a small motor bike. Electricity had not been supplied in the area.

We visited the place on the 13 June, 2001, with a microscope, and made thick blood films from 99 villagers who came to see us for free blood smearing for malaria. Five cases (12 y. o. male, 15 y. o. male, 42 y. o. female, 44 y. o. female, 65 y. o. female) showed positive slides out of the 99, which was considered to be very high in number as could be obtained in a single day survey. They said they were sleeping in the mosquito nets, but that no spraying had been

done in the area. They were taking no counter measures against mosquito bites when they were working in rubber woods in carly morning. The main vector in the area was reported to be *Anopheles minimus*.

The large-scale migration and resettlement of populations from malaria endemic areas into receptive areas were big problems in many parts of the world, however, on the contrary, a movement induced by rural impoverishment influenced by the over population from a non-endemic area to highly endemic areas will also affect the dynamics of the disease badly.

## A－6) RECRUDESCENCE IN VITRO AFTER D-SORBITOL TREATMENT OVER NINE DAYS

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Drug treatment of falciparum malaria is occasionally followed by a recrudescence. Recrudescences have been thought to occur due to drug resistant prasites or due to a low plasma concentration of drug. There have been several reports of discrepancy between in vivo and in vitro resistant tests. Recrudescent parasites obtained from patients were sensitive in the in vitro test. Those reports imply parasites survive drug treatment in some manner other than drug resistance.

Inactive parasites may escape from the effect of drug. More than $1.2\times10^{9}$ erythrocytes infected with cloned parasites were exposed to D-sorbitol treatment 12 hourly for nine days, then maintained in standard medium. D-sorbitol treatment destroys trophozoite and schizont infected erythrocytes and reduces parasite numbers to less than 1/500 per one cycle (48 hours). Parasites reappeared from 4 to 6 weeks after the treatment and multiplied. Erythrocytes infected with recrudescent parasites were as vulnerable to D-sorbitol treatment as those were before recrudescence experiments.

As D-sorbitol treatment causes 500-fold reduction in parasite numbers per cycle, parasite numbers are presumed to be decreased to less than $1/500^{4.5}$ ($=1.4\times10^{-12}$) for nine days. This reduction is far beyond the initial parasite number. Ring-form infected erythrocytes are resistant to the treatment. The results suggest that small percentage of parasites may remain at ring-form stage, so that they survive the treatment. And after treatment they are activated and lead to an observable infection.

## A－7) CHARACTERISTICS OF CHOLERA TOXIN-PRODUCING VIBRIO CHOLERAE STRAINS ISOLATED FROM SEAFOOD IN MALAYSIA

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International spread of enteric infections is becoming a very important issue since the movement of humans and foods across the international borders is increasing year by year. It is known that cholera is endemic in the Bengal area, but cholera outbreaks have been reported in the surrounding areas. We have been studying whether the causative agent of cholera is widely distributed in the environment of such areas surrounding the Bengal area. *Vibrio cholerae* O139 emerged in the Bengal area in 1992 and caused a big outbreak. We previously isolated cholera toxin-producing strains of O139 serotype from the seawater in Malaysia. We therefore examined the seafoods retailed in this country in this study. We purchased and examined the fresh seafoods at 10 sampling sites between 1998 and 1999. As the result,

20 strains of *V*. *cholerae* carrying the *ctx* gene were isolated from shrimps, squids, crabs, and shellfish. They belonged to serotypes O1 Ogawa (one strain), O139 (14 strains), and R (rough, but their *rfb* genes being of the O1 type, 5 strains). All the *ctx*-positive strains produced cholera toxin in vitro and possessed the *tcpA* gene of El Tor type. The *ctx*-positive strains of O1 and O139 serotypes had TLC (toxin-linked cryptic) element, but those of R serotype did not. The R-serotype strains were similar to the clinical O1 Ogawa strain isolated in Kelantan in 1999 because they were negative for the TLC element and shared the same antibiogram. We previously found similar clinical strains in southern Thailand up until March 1998. We compared the environmental strains by various DNA fingerprinting techniques along with the clinical strains isolated in India, Bangladesh, and Malaysia. The pulsed-field gel electrophoresis method gave the highest resolution and the results indicated that environmental O139 strains may have been introduced from India in 1993 and persisted in Malaysian environment since then and that environmental strains of O1 and R serotypes may have served as the source of cholera in Malaysia.

## A－8) FLAGELLA FILAMENT PROTEIN (FliC) INDUCES HUMAN β-DEFENSIN-2 (hBD-2) PRODUCTION IN HOST CELLS

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Antimicrobial peptides such as human β-defensins are crucial for host defense at mucosal surfaces. Expression of defensin genes is regulated in response to infection and inflammation. To evaluate the role of defensin in *S. enteritidis* infection, bacterial factors responsible for induction of human β-defensin-2 (hBD-2) mRNA expression in Caco-2 human carcinoma cells were determined. *Salmonella enteritidis* supernatants of these strains induced hBD-2 mRNA expression in Caco-2 human carcinoma cells. The supernatant factor was heat-stable and proteinase-sensitive. Using luciferase as a reporter gene for a ～2.1 kb hBD-2 promoter, the hBD-2-inducing factor in culture supernatant of *S. enteritidis* was isolated. After purification by anion exchange and gel-filtration chromatography, the hBD-2-inducing factor was identified as a 53-kDa monomeric protein with the amino-terminal sequence, AQVINTNSLSLLTQNNLNK, which is identical to that of the flagella filament structural protein (FliC) of *S. enteritidis*. Consistent with this finding, the 53-kDa protein reacted with anti-FliC antibody, which prevented its induction of hBD-2 mRNA in Caco-2 cells. In agreement, the hBD-2-inducing activity in culture supernatant was completely neutralized by anti-FliC antibody and the lysate with recombinant FliC but not control lysate has hBD-2-inducing activity in culture supernatant.

FliC did not enhance luciferase activity in Caco-2 cells transfected with control luciferse-reporter plasmid (pGL3), or hBD-2 reporter plasmids without NF-κB site (pGL3-197 and pGL3-938mt), consistent with involvement of NF-κB in the induction of hBD-2 by FliC. In gel retardation analyses, FliC increased binding of NF-κB (p65 homo dimer) to hBD-2 gene promoter sequences. These data show that S. enteritidis FliC induces hBD-2 expression in Caco-2 cells via NF-κB activation, and thus plays an important role in upregulation of the innate immune response.

## A－9) ENHANCED EXCYSTATION AND METACYSTIC DEVELOPMENT OF *ENTAMOEBA INVADENS* BY CYTOCHALASIN D

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Excystation and metacystic development are the process by which *Entamoeba* initiates infection. Although the process was described previously, little is known about the mechanism involved. The process of excystation and metacystic development includes movement of the protoplasm of cysts, separation it from the cyst wall, escape of the entire protoplasmic contents through a minute perforation in the cyst wall, and finally production of 8 trophic amoebae. There is, however, no study on the relation of the actin filaments to the excystation and metacystic development of *Entamoeba*. Therefore, we examined the effect of three actin-modifying drugs, cytochalasin D, latrunculin A, and jasplakinolide, on the excystation and metacystic development of *E. invadens* by transfering the cysts to growth medium with the drugs. Cytochalasin D unexpectedly increased the number of metacystic amoebae of *E. invadens* during incubation. Metacystic development, which was determined by the number of nuclei of metacystic amoebae, was faster in the culture with cytochalasin D than without the drug. These results suggest that cytochalasin D enhances the excystation and metacystic development. In contrast, latrunculin A and jasplakinolide inhibited these process. No excystation occurred in encystation medium even in the presence of cytochalasin D, suggesting that growth medium is essential for excystation. Excystation was further enhanced when the cysts were incubated with cytochalasin D before culture in growth medium with the drug. The enhancing effect of cytochalasin D on the excystation and metacystic development was abrogated by jasplakinolide. Thus the results indicate that cytochalasin D, unlike latrunculin A and jasplakinolide, caused enhancement of the excystation and metacystic development of this parasite.

## A－10) INVOLVEMENT OF SIGNALING MOLECULES IN THE GROWTH AND ENCYSTATION OF *ENTAMOEBA INVADENS*

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Signaling molecules are responsible for numerous cellular responces including cell proliferation and defferentiation. There are, however, no studies on signaling for the growth and encystation of *Entamoeba*. Therefore, we determined whether signaling through protein kinase C (PKC), mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (Pl 3-kinase) is involved in the growth and encystation of *E. invadens* by using several inhibitors of these enzymes. Among four inhibitors (staurosporine, chelerythrine hydrochloride, calphostin C, and D-*erythro*-sphingosine) of PKC used, the former two inhibitied the growth and encystation of *E. invadens*. As staurosporine is a potent but not selective inhibitor of PKC, chelerythrine was used for further experiments. Addition of chelerythrine after induction of encystation was also effective for inhibition of encystation. The chelerythrine effect on trophozoites in encystation medium was irreversible, whereas it was reversible in growth medium. All MAPK and MAPKK inhibitors including SB203580, apigenin,forskolin, olomoucine, prostaglandin E2, hypericin, and PD98059 had no effects on the growth and encystation of this parasite. Wortmannin, a specific inhibitor of Pl 3-kinase, also inhibited the encystation of *E. invadens*. Addition of wortmannin after induction of encystation reduced the number of cysts formed. Like chelerythrine, the wortmannin effect on trophozoites in encystation medium was irreversible. These results are the first evidence for participation of PKC and Pl 3-kinase in the growth and encystation of *E. invadens*, contributing to further understanding of *Entamoeba* growth and encystation and their inhibition.

## A－11) BACTERIAL EXPRESSION OF A HUMAN MONOCLONAL ANTIBODY-ALKALINE PHOSPHATASE CONJUGATE SPECIFIC FOR *ENTAMOEBA HISTOLYTICA*

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We previously produced human monoclonal antibody Fab fragments specific for *Entamoeba histolytica* in *Escherichia coli*. For application of these Fab fragments for diagnostic purposes, an experssion vector to produce a fusion protein of Fab and alkaline phosphatase (PhoA) in *E. coli* was designed and constructed. *E.coli* PhoA gene was fused to the 3' terminus of the gene coding the heavy chain Fd region. As human antibody genes, the kappa and Ed genes from one of the previously prepared antibody clones, CP-33, were used. The expressed fusion protein was purified by affinity chromatography using histidine-tag binding resin. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the protein revealed two maior bands with apparent molecular masses of 25 and 75 kDa. To confirm whether these bands represent the kappa chain and the fusion protein of Fd and PhoA, Western immunoblot analysis was performed. As expected, the 25-kDa band was recognized by an anti-human kappa chain goat antibody, and the 75-kDa band was detected by an anti-PhoA rabbit antibody. When the fusion protein of CP33 and PhoA was incubated with formalin fixed trophozoites of *E. histolytica* and developed with substrate, the trophozoites were specifically stained. These results demonstrate that the bacterial expression of a human monoclonal antibody-PhoA conjugate specific for *E. histolytica* is possible and that the antibody can be used to detect *E. histolytica* antigen without the use of chemically conjugated secondary antibodies.

## A－12) THE REGULATORY ROLE OF NITRIC OXIDE (NO) IN PROLIFERATION OF *TOXOPLASMA GONDII* IN MOUSE BRAINS EXAMINED WITH THE TREATMENT OF L-NG-MONOMETHYL- ARGININE AND QC-PCR ASSAY METHOD

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IFN-γ-activated macrophages have been demonstrated as important effector cells *T. gondii* infection via NO-mediated killing mechanism. Previous studies demonstrated that the trealment with L-NG-monomethyl arginine (L-NMMA) of mice increased *T. gondii* numbers analyzed by using direct microscopy and immunofluorescence assay, although these estimation methods of *T. gondii* in tissues seem unreliable because *T. gondii* is an intracellular protozoan. In the present study, we examined the regulatory role of NO in proliferation of *T. gondii* by the use of our own established the quantitative competitive-polymerase chain reaction (QC-PCR) targeting SAG1 gene of *T. gondii*

BALB/c and C57BL/6 mice were intraperitoneally (i. p. ) injected with PBS (as a control) or L-NMMA (175mg/kg), and the mice were i. p. infected with 200 or 400 cysts of *T. gondii.* All mice treated with L-NMMA died within 13 days post-infection. In contrast, 33% of the control BALB/c and C57BL/6 mice treated with PBS and infected by 200 or 400 cysts of *T. gondii* died within 10 to 11 days post-infection and the remainder survived. These data showed that the treatment of L-NMMA reduced the host resistance of both susceptible C57BL/6 and resistant BALB/c mice to tigh dose infection of *T. gondii.* In an attempt to characterizc whether injection of L-NMMA results in enhancement of proliferation of *T. gondii* in brains of infected mice, the number of *T. gondii* in the brains was measured using QC-PCR targeting SAG1 gene 8 weeks after *T. gondii* infection. The number of *T. gondii* in the brains of L-NMMA-treated BALB/c and C57BL/6 mice was significantly higher than that in control mice. These data indicate that the treatment of L-NMMA increased the number of *T. gondii* in brains of BALB/c and C57BL/6 mice. After infection with *T. gondii,* peritoneal macrophages from PBS-treated BALB/c and C57BL/6 mice pro-

duced high levels of NO. Peritoneal macrophages from L-NMMA-treated BALB/c and C57BL/6 mice, however, produced low levels of NO. Thus, L-NMMA blocked NO synthesis of peritoneal macrophages of *T. gondii*-infected mice.

Activated macrophages of *T. gondii*-infected mice down-regulated proliferation of *T. gondii* in the brain and dissemination of *T. gondii* to the brains of infected mice via NO-dependent effector mechanisms.

## A−13) *T. GONDII* INFECTION INDUCES ANTI-HSP70 AUTOANTIBODY IN A SUSCEPTIBLE C57BL/6 MICE BY MODULATING RAG GENES

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**Introduction**: *Toxoplasma gondii* (*T. gondii*) an obligate intracellular protozoan parasite, is an important cause of morbidity and mortality, especially in congenital toxoplasmosis and immunocompromised hosts. We have reported anti-*T. g.* HSP70 antibody as well as anti-mHSP70 autoantibody produced in dominantly *T. gondii*-infected C57BL/6 (B6; a susceptible strain) mice. The mechanism of autoantibody formation in *T. gondii*-infected mice is not well defined. The lymphoid-specific genes, recombination-activating genes (RAGs) catalyze V(D)J recombination. The purpose of this study was to investigate the mechanisms of autoimmunity induced by *T. gondii*-infection.

**Material and Methods**: BALB/c and B6 mice were perorally infected with 5 cysts of Fukaya strain. Control uninfected and infected BALB/c and B6 mice were sacrificed on day 0, 3 and 7 after *T. gondii*-infection. Spleen, MLN, BM and PECs were collected for the determination of the expression of RAG gene. The *T. gondii* numbers in the tissues of uninfected and *T. gondii*-infected BALB/c and B6 mice were analyzed by quantitative competitive-PCR.

**Results**: The level of RAG1 mRNA expression significantly decreased in the spleen and MLN of B6 mice on day 3 and recovered and reached a plateau 1 wk after *T. gondii* infection. Before *T. gondii* infection, the expression of RAG1 was not observed in PECs of B6 mice. One week after *T. gondii* infection, RAG1 and RAG2 expression was induced in PECs of *T. gondii*-infected B6 mice. The level of RAG1 expression in BM of B6 mice gradually increased after *T. gondii* infection. These data indicated that the level of RAG1 expression was transiently down-regulated in the spleen and MLN 3 days after the infection, then a higher level of RAG1 expression was observed in the spleen, MLN, BM and PECs of B6 mice 7 days after *T. gondii* infection. Significant changes in the RAG1 expression in the spleen, MLN, BM and PECs of BALB/c mice was not shown after *T. gondii* infection. On the other hand, the numbers of *T. gondii* in the spleen and MLN of BALB/c and B6 mice reached a peak 7 days after the infection and then decreased. No *T. gondii* was detectable in BM and PECs in BALB/c mice throughout the course of infection.

**Conclusion**: Out data indicated that the expression of RAG was transiently down-regulated in the spleen and MLN but not in BM and PECs of *T. gondii*-infected a susceptible B6 mice and the levels of RAG expression correlated with the *T. gondii* numbers existent in the tissues.

## A−14) A THOUGHT EXPERIMENT ON THE LINK BETWEEN THE RISK OF CONGENITAL TOXOPLASMOSIS AND THE ANNUAL INFECTION RATES

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Toxoplasmosis, an infectious disease induced by *Toxoplasma gondii* (*T. gondii*) an obligate intracellular protozoan, is commonly benigh or asymptomatic when acquired by healthy persons. However, it may give harmful effects to fetus when pregnant women contract a primary infection congenital toxoplasmosis (CT).

The risk of CT reflects the total number of women who acquire a primary infection in their pregnancy. Hence,

it also correlates with that of women who acquire a primary infection in the gestational period: the age equal to or older than 15 years and younger than 45 years. In the present study, we made a theoretical analysis with a simple mathematical model on the total number of women in the gestational period who get a primary *T. gondii* infection, through which we found out the link between the risk of CT and the annual infection rates of *T. gondii*. Considering the differences in behavioral patterns, different annual infection rates, the rate before the gestational period (r1) and that after the period (r2), were applied to out model.

Our present study indicates, in the case that annual infection rate is constant (r1 = r2), 3.67% of annual infection rate maximizes the risk of CT, while the rates vary if it is presumed that r1 is different from r2. Adding to it, changes in r1 would make a shift of the risk curve, while those in r2 would move the location on the same risk curve.

Based on our study, the risk of CT in Japan currently stands st a relatively lower level. However, the study implies that a possible epidemic of *T. gondii* infection could raise the risk steeply. From the standpoint of risk management, public intervention programs are required.

Our study also implies that public interventions for CT could generate different outcomes, depending on whether they target women before the gestational period or after the period. And a successful program in certain area could induce a harmful effect in another. Tailor-made medicine is required in public health policy as well as clinical medicine.

## A－15) PHENOTYPE AND FUNCTION OF MURINE PERITONEAL CAVITY MACROPHAGE DERIVED DENDRITIC CELLS

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We describe a two-step culture system using peritoneal macrophages from P30 (SAG-1) transgenic mice as a source of dendritic cell (DC) progenitors. The proliferative capacity of the progenitors was smplified in the first step of the culture (day 0-7) using a combination of early cytokines: interleukin 4 (IL-4); and granulocyte-macrophage colony-stimulating factor (GM-CSF). The second step of the culture started at day 7 with the removal of early growth factors to allow differentiation and final maturation of DC during 2 days of culture with interferon gamma (IFN-γ) plus Toxoplasma lysate antigen (TLA) as maturing agents. Here, we show that the resulting DC population exhibits typical dendritic cell morphology, expresses MHC class II and co-stimulatory molecules CD80 at higher levels, consistently synthesize IL-12, efficiently stimulate T cell responses in vitro using two way mixed lymphocyte reaction (MLR) and are able to present soluble antigen to $CD3^+$ spleen T cells.

## A－16) GROWTH INHIBITORY EFFECTS OF TRICLOSAN AGAINST *BABESIA* SPP. PARASITES

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The antimicrobial biocide triclosan ｜5-chloro-2 (2.4-dichlorophenoxy) phenol｜ has been reported recently to inhibit the growth of *Plasmodium falciparum in vitro* and *P. berghei* in mice (Nature Medicine, 7: 167-173, 2001). In the present study, we have evaluated the growth inhibitory effect of triclosan against the hemoprotozoan, tick-transmitted parasites, *Babesia egui B. caballi* and *B. bovis* The growth inhibitory tests were carried out *in vitro* following the method of lgarashi et at. (Expt. Parasitol., 90: 290-293, 1998). Different concentrations of triclosan were added to cultures containing an initial 1% parasitemia of *Babesia* parasites in 24-well plates and incubated at 37℃. The medium was changed daily and the percent parasitemia was monitored on Giemsa-stained blood smears for five

days. Parasite growth of *B. egui* and *B. caballi* was inhibited at drug concentrations of 20-200 μg/ml, while growth inhibition of *B. bovis* was noted at 100-500 μg/ml. Initial findings in *in vitro* studies are encouraging and suggest the use of triclosan as a potential drug against equine and bovine babesiosis. To further determine the efficacy of triclosan in vivo, we have started studies on its effect on *Babesia rodhaini*-and *B. microti*-infection in mice.

## A−17) GEOPATHOLOGICAL STUDY ON MALIGNANT TUMORS IN WESTERN KENYA BETWEEN 1979 AND 1998

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An ethnogeographic variation in the frequency of different types of tumors is mainly attributed to the interplay of varied causative factors such as exposure to ultraviolet light, chemical carcinogens, oncogenic viruses, genetic factors, and cultural practices among various populations. This study analyzed histopathologic specimens in western Kenya for 20 years during 1979 to 1998 with a focus on the following objectives; to examine ethnogeographic distribution and to relate the tumors to putative environmental causative factors. The most common malignant tumor was uterine cervix cancer, which is widely distributed in this area, followed by malignant lymphoma, including Burkitt's lymphoma, which is more prevalent among the Luo around the Lake Victoria, and squamous cell carcinoma of the skin among the Kalenjin in Tropical Highlands. In addition, several malignant tumors such as Burkitt's lymphoma, Kaposi's sarcoma, penile cancer, squamous cell carcinoma of the urinary bladder showed characteristic ethnogeographic distributions. These findings suggest that environmental factors, including oncogenic viruses, and cultural practices seem to play a main role in the development of malignant tumors in western Kenya.

## A−18) SYNERGISTIC EFFECT OF TWO SUSCEPTIBLE GENETIC MARKERS, HLA-B5*0101 AND IL-13P-A/A, ON THE DEVELOPMENT OF POST-SCHISTOSOMAL LIVER FIBROSIS

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In 1995, over 100,000 patients have been estimated to be infected with *Schistosoma japonicum* in Jiangxi province. One of the most serious complications is post-schistosomal liver disease that develops within several years after the infection. Schistosomal egg antigen specific CD4+ T cells play a major role in the formation of granuloma through Th2 type cytokine production in experimental schistosomiasis mansoni. To identify the host genetic factors affecting the prognosis after infection, we have examined DNA polymorphism of 192 unrelated past patients who were living in the endemic area named Yushan, Jiangxi, including 36 individuals with no fibrotic change in the liver (Grade O) and 156 patients with typical fibrotic change (Grade I through Grade III), diagnosed by ultrasonography (Cairo, 1991). After extensive analysis of HLA-class II region and polymorphic immune response related genes (TNF-α promoter, IL-4R, IL-4 promoter, IL-4, IL-13 promoter, IL-13, region), the HLA-DRB1*1101 and HLA-DRB5*0101, IL-13 promoter A / A genotypes were revealed to be associated with protection and susceptibility to fibrosis. Moreover, HLA-DRB5*0101 and IL-13P-A / A both of which were susceptible markers to fibrosis were synergisti-

cally elevated the odd's ratio. This effect was considered to be mediated though IL-13 production up-regulation in the antigen specific CD4+T cells stimulated by HLA-DRB5* 0101.

## A－19) A SURVEY ON ANTIGENEMIA OF *WUCHERERIA BANCROFTI* AND URINE ANTIBODY TITER IN RURAL COMMUNITIES IN NEPAL

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In order to determine the prevalence of the bancroftian filariasis in rural communities in Nepal, serum and urine samples from inhabitants in two rural villages (Judigaun, Kotyang) were examined. Serum and urine samples of 244 inhabitants in Judigaun, which locates 30 km from Kathmandu, were collected in 2000, 2001. Stored serum and urine samples collected from inhabitants in Kotyang in October 1998 at before survey were also used. Serum samples were examined for *W. bancrofti* antigens by using Og4C3 assay. Immunoglobulin G4 against filarial antigen in the urine samples were measured by using filarial antigen coated 96-well micro titer plates and peroxidase conjugated second antibodies to human IgG4. Among 238 serum samples and 244 urine samples from Judigaun people, 60 (25.2%) serum samples and 124 (50.8%) urine samples were positive with regards to *W. bancrofti* antigenemia and filarial IgG4 titer respectively. Among 117 serum samples and 364 urine samples from Kotyang people, 18 (15.4%) serum samples and 90 (24.7%) urine samples were also positive with antigenemia and IgG4 titer respectively. These results show that rural area are also endemic of *W. bancrofti* and more detailed study will be expected.

## A－20) MATHEMATICAL MODEL FOR THE TRANSMISSION OF LYMPHATIC FILARIASIS AND ITS APPLICATIONS

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Our study aims at evaluating the effect of vector control on the prevalence of Iymphatic filariasis caused by *Wuchereria bancrofti* using computer simulations based on the stochastic transmission model, and especially it is focused on the continuous effect for a post control period.

In Pondicherry, South India, Vector Control Research Centre had carried out the integrated vector control program against filariasis and malaria for five years (1981-1985), and reported a substantial decrease in both vector population and the transmission index for *W. bancrofti* during the period of vector control. We improve the transmission model proposed by A. P. Plaisier *et al*. (1998) to be fitted for a prevalent situation in Pondicherry on the basis of pre-control epidemiological data there. The population dynamics of mosquito vectors has been simulated by the carrying capacity model of the larval mosquitoes environment.

Our simulations show that in the vector control area the rate of microfilaremia in human population is decreasing slowly while the mean number of L3-larvae carried by mosquito population is decreasing in an early period of a

control project. For a post control period, they also show that the prevalence revives in a several years and is beyond the initial prevalence level because of a decline of immunity level if an effect of vector control looses thoroughly, but the prevalence stays at low level for a long time if a continuous effect remains at least 10%. We conclude that in a post control period keeping of a little continuous effect contributes to control or elimination of lymphatic filariasis.

## A－21) A TRIAL TO DETECT ANTI-*B. PAHANGI* IGG4 IN URINE FROM CHILDREN UNDER 5 YEARS OLD IN ORDER TO EVALUATE FILARIASIS CONTROL MEASURES

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The global program to eliminate lymphatic filariasis has been launched. For the program, it is essential to find endemic areas and evaluate the effect of control measures. Surveillance of infectious status of young children will be useful for the evaluation, since successful control prevents new infections and children born after the control measures are expected to harbor no filarial parasites.

We developed a sensitive and specific ELISA for bancroftian filariasis using urine samples. In this study, we applied the ELISA method to evaluate filarial infections among young children in a bancroftian filariasis endemic area.

**Subjects and Methods**

Urine samples were collected in Matara District, Sri Lanka from 206 children under 5 years old including 38 infants. Samples from their mothers and / or fathers (301 samples) were also collected. Urine collection bags (Atom Pediatric Urine Collector) were used for small children and infants. Anti-*Brugia pahangi* IgG4 antibodies in urine samples were measured by ELISA.

**Results and Discussion**

Urine samples from small children could be collected easily and safely. Filaria antigen specific IgG4 antibody were positive in 10.5% (4/38) of infants (≦1 year old), 7.5% (3/40) of the age group 1-2 years, 2.3% (1/43) of the age 2-3 years, 22.5% (12/47) of the age 3-4 years and 26.3% (10/38) of the age 4-5 years. A remarkable increase in the positive rate was observed at age 3 years old and above, indicating that the filarial infections occur very early in life in this area. The positives in infants have antibodies transferred from their mothers.

These results show that the measurement of filarial antigen specific IgG4 antibodies in urine samples from children is easy and useful for evaluation of the parasite control program.

## A－22) RESISTANCE OF *BLASTOCYSTIS HOMINIS* TO METRONIDAZOLE

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*Blastocystis hominis* has been found on numerous occasions in the human intestinal tract. Although most cases of *B. hominis* infection are symptom-free, symptomatic infections have been reported, and the pathogenicity of this unicellular organism remains controversial. When a *B. hominis* infection is diagnosed, chemotherapy is usually prescribed. At present, the drug of first choice is metronidazole. We used metronidazole to treat 15 infected individuals whether or not they were symptomatic. Of the 15 infected individuals, 11 proved refractory to treatment even though large doses of metronidazole were administered. Because none of our surveyed individuals were immuno or otherwise

deficient, any GI symptoms were believed to have been due to the infection with *B. hominis*. It is informative that all symptoms disappeared after treatment. To elucidate the causative factors of the chemotherapeutic failure, we monitored the in vitro effects of metronidazole on *B. hominis* isolates obtained from the refratory individuals both before and after treatment. Metronidazole was effective in inhibiting growth of control isolates obtained from non-treated cases and the reference strain (Nand II), although it took 4 hours to manifest efficacy. In contrast, the refractory *B. hominis* isolates showed a slightly increased resistance in vitro to metronidazole. On the basis of the present results, it seems that treatment failure may have resulted from insufficient exposure to metronidazole and that *Blastocystis* may acquire a degree of resistance after initial exposure.

## A－23) LONG TERM ERADICATION RATE OF IVERMECTIN THERAPY FOR STRONGYLOIDIASIS ON YORON ISLAND, KAGOSHIMA PREFECTURE

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In our present study, we investigated the epidemic of *Strongyloides stercoralis* on Yoron Island, Kagoshima Prefecture. Furthermore we assessed the long term eradication rate of ivermectin therapy for chronic strongyloidiasis and adverse effects of the drug. Yoron Island is situated in the southernmost area of Kagoshima Prefecture. Of the 611 persons examined by using agar plate culture method, 64 (10.5%) were found *Strongyloides*-positive. By age, the positive rate was highest in 60s (16.0%), following by 70s (13.8%), 80s (12.5%), 50s (6.9%) and 40s (6.5%), Of those who were found *Strongyloides*-positive, 32 persons (17 males and 15 females) desired to have treatment. HTLV-1 antibody positive rate was 25.0% (8 / 32), of which males were 23.5% and females 26.7%. To each patient, approximately 100 $\mu$g/kg of ivermectin was administered before breakfast. The same treatment was repeated after 2 weeks. During the period of administration of ivermectin, Liver dysfunction was observed in one patient. However, it was of mild with only slight increases in ALT, and no particular treatment was required. As subjective adverse effect, a feeling of vertigo was noted in one patient after administration of 2 courses. In this case, too, no particular treatment was necessary. In the 32 patients who received treatment, the eradication rates in 2 weeks and 4 weeks after first treatment were 100% (32/32 patients) respectively. In the follow-up investigation conducted one and four year later, 2 cases of recurrence (30/32, 26/28) were found respectively. Both of the 2 patients were HTLV-1 antibody positive.

Ivermectin is being used widely all over the world as a drug for Onchocerca volvulus, and it shows high anthelmintic activity against *S. stercoralis* too. Ivermectin showed excellent anthelmintic effects with mild toxicity. We therefore consider ivermectin is the most useful agent for the treatment of chronic strongyloidiasis at present.

## A－24) PREVALENCE OF OXYURIOSIS AND OTHER HELMINTH AT THE SCHOOL OF MOUNTAINEOUS REGION OF NEPAL: INFLUENCE BY TARGET SELECTED AND BLANKET TREATMENT

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Helminth is one of major cause of economical loss in developing world. JAITI (Japan Agriculture In-service Training Institute) Basiphant School was established in 1993 at mountainous region located about 80 km southwest of capital city Kathmandu in Nepal. The school children were treated by mebendazole twice a year since 1993. We

have started school health project in 1998. Two hundred forty school children aged six to twenty first were the target. The prevalence of helminth was 60% at initial time in 1998 and considered that the result was contributed to blanket treatment. We examined the stool and selected positive cases and treated them by albendazole but not any medication to negative cases. One years interval showed high prevalence of helminth over 80%. The result suggested high re-infection rate and necessity of blanket treatment. We have started blanket treatment by albendazole twice a year then after two years later in 2001 the prevalence was decreased to 58%. But the prevalence of Enterobius infection using scotch tape method was 43% in 1999 and 52% in 2001. This data indicated several speculation to re-consider the strategy of the project as follows: 1. Target selected treatment is not an adequate method to control helminth infection in the school. 2. Re-infection rate is supposed to be high and even by six months blanket treatment may not be expect to control the prevalence less than 50%. 3. Health education programme involving families of children has not been performed yet. The infection cycles in each family and community may contribute high re-infection rate at the school. 4. Increased prevalence of Enterobius infection may due to improved technique by children to collect the specimen. 5. Treatment performed twice in a year by albendazole does not decrease the prevalence of Enterobius infection. This may due to high infectivity of Enterobius ova and poor hygienic condition particular clothes and bed linens. 6. Improving hygienic condition by health education may only be able to decrease the prevalence of helminth infection less than 50% combined with blanket treatment in the school. We are planning to involve families of children then community to health education programme in near future.

## A－25) A SURVEY ON ALLERGY, PARASITIC INFECTION AND NUTRITIOUS STATUS IN SCHOOL CHILDREN IN VIETNAM

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A survey on allergy, parasitic infection and nutritious status in school children in Hanoi, Vietnam was carried out to know relationships among allergy, parasitic infection, and nutritional status. Eighty eight children with an average age of 10.6 years old were examined. For intestinal parasitic infection their feces samples were examined using Kato-Katz technique. To survey their allergic diseases or symptoms all the children were done by use of a questionnaire. Daily food intake was assessed by the '24 hour recall' method. Their weight and height were measured. Anthropometric indices were analyzed using the standard US National Center for-Health Statistics charts and tables. Body mass index (BMI) was also calculated. Blood samples were collected and analyzed for serum IgE, total protein, albumin and lipid in plasma.

Results: 1) Prevalence of parasitic infection (83.0%) was very high. *Trichuris trichura* was a most dominant parasite (80.7%). 2) Only 9 children had a history of allergy. Six out of them were infected with parasitic infection and no relationship between the presence of allergy and parasitic infection was observed. 3) The level of total IgE in serum was significantly higher in children with parasite (474.4 IU/m*l*) than that in those without parasite. 4) Nutritional status of chidren examined was poor regardless of parasitic infection. Thirteen and sixteen children were considered to have malnutrition by weight-for-age and height-for-age analysis respectively. 5) The mean serum level of total protein, total cholesterol, triglyceride etc were all within the normal range. 6) For nutritional intake, the mean energy was 1413.9Kcal, showing about 70% of recommended dietary allowance in Vietnam. No difference in nutritional intake such as protein, lipid and carbohydrate was observed between the children with and without allergy and parasitic infection.

Conclusions: In Vietnam the prevalence of intestinal parasitic infection was high but the number of children with a history of allergy was few. Nutritional status of children was very poor regardless of parasitic infection. No relation-

ships between allergy and parasitic infection, and between allergy and nutritional intake were observed.

## A－26) HIV-1 SUBTYPING IN ZAMBIA

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**Introduction**: Sub-Saharan Africa has the highest HIV prevalence and AIDS burden. The ultimate goal of controlling the HIV-1 pandemic is to develop safe and efficacy vaccines. However, the relatively high level of genetic divergence between the group M clades has led to the hypothesis that multiple vaccines against HIV-1 may have to be made against the different subtypes of the virus. Sequence information on most of the nine subtypes is currently limited, suggesting that more information will be needed if subtype specific vaccines are to be produced. We are currently carrying out a nation-wide molecular epidemiological study that seeks to access the distribution of HIV-1 subtypes in Zambia. **Objective**: To document the HIV-1 subtypes circulating in Lusaka, Luapula and Northern provinces in Zambia. **Methods**: Two hundred and thirty samples collected from antenatal women in seven clinics in Lusaka province were initially screened for HIV using antibody assays. Twenty-five and eight HIV-1 seropositive whole blood samples were collected from provincial headquarters of Northern and Luapula provinces respectively. Molecular epidemiology of HIV-1 subtypes was investigated by direct sequencing of PCR products of *gag* p17 and *env* gp120 C2-V3-C3 fragments and phylogenetic analysis was done. **Results**: Sixty-three samples from Lusaka province were HIV-1 positive. The number of HIV-1 *gag* p17 and *env* gp120 sequences obtained per province were 56 for Lusaka, 20 for Northern, and 8 for Luapula. Thus a total of 84 sequences from 92 (91%) HIV-1 positive samples were obtained. HIV-1 *gag/env* subtype C per province were 51 (91%) for Lusaka, 20 (100%) for Northern, and, 8 (100%) for Luapula. One (1.8%) HIV-1 *gag/env* subtype A-like, one (1.8%) HIV-1 *gag/env* subtype G-like, and one (1.8%) A/C and one (1%) D/C recombinants were detected from Lusaka samples. A high conservation of the CTL peptide epitopes was observed among HIV-1 *gag* and *env* subtype C-like viruses. **conclusions**: HIV-1 subtype C is predominant in Lusaka, Luapula and Northern provinces. Two new HIV-1 *gag/env* subtype A-like and subtype G-like have been identified for the first time in Zambia in this study suggesting that more subtypes could be in existence. Two suspected recombinants, A/C and D/C, have also been detected in this study. We are now doing full length genome analysis to determine the nature of recombination of these samples. Some of the more conserved CTL epitopes among different HIV-1 subtypes should be considered in vaccine constructs in order to have cross clade immune responses. There is need for a continued work of HIV subtyping to enhance the efforts being made towards the development of HIV-1 vaccines.

## A－27) MORECULAR EPIDEMIOLOGY OF HIV IN MYANMAR: EMERGENCE OF NEW FORMS OF HIV-1 INTERSUBTYPE RECOMBINANTS

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**Background**: UNAIDS estimated that 530 thousands people has been infected in Myanmar (as of the end of 1999) since the HIV-1 was introduced in 1989. Highest HIV prevalence is observed among injecting drug users (IDUs) (63%) and female commercial sex workers (fCSWs) (38%). In order to study the molecular epidemiology of HIV-1 in Myanmar, we investigated the molecular nature of circulating HIV strains and the interrelationship of the epidemic with those in surrounding countries.
**Methods**: The nucleotide sequences of various segments in HIV-1 genomes were determined and subtyped for 84 specimens from Mandalay, Central Myanmar. The near full-length sequences of plausible recombinant HIV-1 isolates were determined after PCR-cloning and were subjected to the phylogenetic and recombination breakpoints analyses.
**Results**: Phylogenetic analyses based on *gag* (p17) and *env* (C2/V3) sequences detected 13 subtype C (16%), in addition to the previously identified subtype B' (Thai-B cluster within subtype B) (19/84, 23%) and CRF01_AE (37/84, 44%) that are likely to be originated from neighboring Thailand. The majority of subtype C in Myanmar belongs to the India-China cluster of subtype C. Notably, the remaining 15 specimens (18%) showed discordance between *gag* and *env* subtypes, including 4 *gag* B'/*env* C (B'/C), 3 C/B', C/E and B'/E, and 2 E/B'. These discordant specimens were found most frequently among IDUs (10/34, 29%). The analyses based on near full-length HIV-1 sequences revealed the unique chimeric structures between co-circulating subtypes of B', C and E. in four isolates.
**Conclusions**: The data suggest that multiple subtypes of B', C and CRF01_AE are co-circulating in Central Myanmar, leading to the evolution of new forms of HIV-1 intersubtype recombinants. The multiple occurrences of the recombinants with unique genome structures suggested on-going recombination between these three circulating subtypes in Central Myanmar. Our finding would provide information critical for future vaccine strategies aimed at this particular area in Asia.

## A－28) TRIALS OF DNA VACCINE TO JAPANESE ENCEPHALITIS VIRUS (JEV): EXPRESSION OF JEV-ENVELOPE PROTEIN IN MAMMALIAN CELLS

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Japanese encephalitis virus (JE) is a serious mosquito-borne viral disease of major public health important in Asia. Recently it has been reported that DNA vaccine is effective to protect the virus infection including JE virus. Expression level of transfected plasmid appears to be related with the efficacy of DNA vaccine. Here we constructed two kinds of expression vectors, pJE and pJME for JE envelope (E) protein and compared the expression level in several mammalian cells. Both expression vectors containing FLAG tag were transfected with liposome (DMRIE-C reagents, GIBCO) into cultured mammalian cell lines, i.e. HepG2 and KN73 derived from human liver, COS-1 and Vero derived from monkey, and BHK cells. To detect expression proteins, Western blot using anti-FLAG or anti-E sera was performed. In the transfected cells, 72kDa (prM+E) and 53 kDa(E) protein were expressed under the control of CMV promoter. The expression of E protein in the transfection with pJME was usually higher than that of pJE. It is likely that prME protein is cleaved to E protein in the cells transfected with plasmid. In addition, expression level was dependent on the amounts of DNA transfected, and different among the cell lines. Protein expressions in HepG2, KN73, COS1 were relatively higher than those in Vero and BHK cells. The expression efficacy of E and other proteins may be influenced by the host factors including peptidase. It is important to check the features of protein expression for the development of an effective DNA vaccine.

## A－29) EVALUATION OF DENGUE ANTIGEN DETECTION KIT

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Dengue fever (DF) and dengue hemorrhagic fever (DHF) have been a serious health problem in most of the tropical and subtropical countries in the world. Dengue virus is not endemic in Japan, however, there are imported cases. According to the Japanese new infectious disease control law, which became effective on 1st April 1999, DF/DHF is one of the infectious diseases to be reported by physicians.

We perform reverse transcriptase polymerase chain reaction (RT-PCR) as one of the laboratory diagnostic methods for virus infection, especially with sera obtained duaring a febrile period. Dengue antigen detection Kit (Globio) has recently been on the market. We examined this test kit for sensitivity and specificity in comparison with RT-PCR.

77 serum specimens were collected from dengue-suspected febrile patients and sixty serum specimens (as negative controls) were collected from exanthema subitum patients who had never been to dengue epidemic area. They were tested by RT-PCR, IgM capture-ELISA and Dengue Antigen Detection Kit (Globio).

77 serum specimens collected from febrile patients were divided into three groups by the results of RT-PCR and IgM capture-ELISA. Twenty-two samples in 1st group were RT-PCR (+) and IgM capture-ELISA (-). Twenty-five samples in 2ndgroup were RT-PCR (-) and IgM capture-ELISA(+). Thirty samples in 3rd group were RT-PCR (-) and IgM capture-ELISA(-). The results obtained by the antigen detection kit were as follows. ①Five (23%) of the 22 samples in first group were determined to be negative. ②Eight (32%) of the 25 samples in second group were determined to be positive. ③Six (20%) of the 30 samples in third group were determined to be positive. More over, 5 (8%) of 60 sera from exanthema subitum patients were determined to be positive.

These results demonstrated that this kit has non-specific reaction and the sensitivity is not as high as RT-PCR. However, if high specificity and sensitivity are established by some modification, this technique may be useful for rapid diagnosis, in certain facilities.

## A－30) BIOLOGICAL CHARACTERISTICS OF DENGUE 2 VIRUSES ISOLATED FROM PATIENTS WITH DIFFERENT SEVERITIES IN BAN HOM COMMUNE, RURAL AREA OF VIENTIANE, LAO P. D. R.

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Dengue outbreak occurred in a rural commune (Ban Hom, 4 $km^2$) Vientiane, Lao PDR in 1994. Five strains of dengue virus type 2 (DEN2) were isolated from patients with different clinical severities in this area within 15 days. In order to identify possible viral factors in the pathogenesis of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), biological characteristics of these 5 strains were compared.

Each strain passaged 3 times in the mosquito cell line C6/36 was tested for mouse-neurovirulence, plaque size and virus production in cultured cells, fresh peripheral blood mononuclear cells (PBMC), and dendritic cells. Basic characteristics of the 5 isolates are ; (name: severities, primary or secondary infection, sampling date) 1) UOFP: Fever of unknown origin, primary, June 1, 2) UOFS: Fever of unknown origin, secondary, June 1, 3) DF: dengue fever, secondary, June 15, 4)DHF: DHF grade I, secondary, June 15, 5) DSS: DHF grade III, secondary, June 15. Prototype DEN 2 (New Guinea C strain) was used as a control.

Mouse neurovirulence was determined by intracerebral inoculation in 3-day-old ICR mice with each strain at $10^4$focus forming units (ffu). Death rates at 3 weeks after inoculation of DSS, UOFP were 0%, DHF 17%, UOFS 26%, DF 38%, NGC 100%. Plaque size in C6/36 cells were; UOFP, DHF(7.56-7.95 mm), UOFS, DF, DSS (5.13-5.30 mm). Plaque formation in LLC-MK2 cells was not observed for any of the 5 isolates but for NGC (1.52 mm). The production of virus particles in culture fluid was determined in ffu/

m*l* using PAP staining method. C6/36 cells could provide efficient replication of all isolates ranging 3-9x $10^7$ ffu/m*l* and NGC, 6x$10^8$ ffu/m*l*. LLC-MK2 cell line showed significantly lower production for every isolate (2x$10^3$-3x$10^4$ ffu/m*l*) than C6 / 36. The production of virus particles were not detected in infected culture fluid of PBMC for any of the 5 isolates, while infection of all isolates and NGC in PBMC was confirmed by indirect immunofluorescenece reaction. In immature dendritic cells -like cells generated from PBMC by addin IL-4 and GM-CSF to culture fluid, all 5 isolates could produce virus particles efficiently ranging 1.2-1.8x $10^5$ ffu/m*l* and have peaks of growth curves at 34 hours after infection.

All biological characteristics studied here did not correlate with the clinical severity of the patient from whom the virus was isolated.

## A－31) COMBINED DETECTION AND GENOTYPING OF CHIKUNGUNYA VIRUS BY A SPECIFIC REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

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A reverse transcription-polymerase chain reaction (RT-PCR) was developed for the detection of *chikungunya* virus (CHIK) infection. Based on the non-structural protein 1 (nsP1) and glycoprotein E1 (E1) genes of CHIK, two primer sets were designed. Total RNA were extracted from the cell culture fluid of *Aedes albopictus* C6/36 cells inoculated with the S27 prototype virus, isolated in Tanzania in 1953, and the Malaysian strains (MALh0198, MALh0298 and MALh0398), isolated in Malaysia in 1998. For both sets of RNA samples, the expected 354 and 294 base pair (bp) cDNA fragments were effectively amplified from the nsP1 and E1 genes, respectively. The sensitivity of the RT-PCR was approximately 5 plaque-forming units (PFU) for the nsP1 primer set and 50 PFU for the E1 primer set when applied to the S27 strain. RT-PCR products from both S27 and Malaysian strains were sequenced and compared. For the 314 bp-long sequences of the nsP1 gene, the nucleotides were 96.8% to 97.1% homologous and the amino acid sequences deduced for the S27 and Malaysian strains were identical. For the 257 bp-long sequences of the E1 gene, the S27 prototype and the Malaysian strains showed a 96.5% and 97.6% homology for the nucleotide and the amino acid sequences, respectively. Phylogenetic analysis was conducted for the Malaysian strain and other virus strains isolated from different regions in the world endemic for CHIK, using partial E1 gene sequence data. The Malaysian strains isolated during the epidemics of 1998 fell into a cluster along with other members of the Asian genotype.

## A－32) A QUESTIONNAIRE SURVEY FOR DENGUE CONTROL IN BLITAR MUNICIPALITY, EAST JAVA PROVINCE, INDONESIA: AN EVALUATION FOR BETTER COMMUNITY EDUCATION APPROACH

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The vaccine against Dengue virus infection has not been produced so far. The only method to prevent dengue fever (DF) and dengue hemorrhagic fever (DHF) is the control of vector mosquitoes. In line with these facts, the Ministry of Health of Republic Indonesia has been carrying out an anti-DHF-campaign of mosquito control both via health centers and mass media. In order to assess the effectiveness of the campaign, a questionnaire survey was conducted in a community. The objectives of the study were (i) to explore the knowledge, attitude and practices of the people about DHF control and to identify the relating factors, and (ii) to assess the performance of DHF anti-DHF-campaign. The

survey was conducted in Blitar municipality, East Java Province, Indonesia. By using multistage random sampling, 198 households from 4 villages (*kalurahan*) were selected. The head of a household or the other member, such as wives and children, of the household was interviewed by using semi-open questionnaire (guided interview). Analysis was made using SPSS Release 10 (SPSS Inc.). The items in the questionnaire were sex, marital status, position in their family, age, education level, occupation, questions about the source and the mean of dengue information and questions to determine their knowledge, attitude and practice for the control of mosquitoes.

Out of 198 respondents, the heads of the household were 103 consisting of 87 males and 14 females, wives were 74 and children were 21. The mean age of the respondents was 44.0 with a range of 13-77. Fourteen did not graduate from any school, 83 graduated from elementary school, 41 from junior high school, 54 from high school and 6 graduated from college or university. Twenty four were farmers, 37 were merchants, 22 were civil servants, 22 was working at private company, 33 were unskilled workers and 60 were unclassified.

Although most of the respondents (184/198) were aware of dengue fever (DF) and dengue hemorrhagic fever (DHF), a lot of heterogeneity was observed on their knowledge, attitude and practice. The factors that associate with the heterogeneity will be demonstrated and discussed.

## B－1) PURIFICATION OF RECOMBINANT TRYPANOSOME ALTERNATIVE OXIDASE

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African trypanosoma is a parasite, which causes African sleeping sickness of human and nagano disease of cattle. Because at present drugs against trypanosomes have strong side effect and are not effective for chronic patients, a new effective drug is awaited now. We found that ascofuranone isolated from *Ascochyta visiae* inhibits specifically mitochondrial respiration of blood stream form of *Trypanosoma brucei brucei* at very low concentration. In addition, we found that injection of ascofuranone into infected mice made blood stream form of *T.brucei brucei* die out.

It is said that the target of ascofuranone is cyanide resistant terminal oxidase located at mitochondrion of blood stream form of *T.b.brucei*. This enzyme is referred to as TAO (trypanosome alternative oxidase), and functions ubiquinol oxidase unlike cytochrome *c* oxidase of mitochondrion of mammal. Because mammals don't have TAO that is essential for reoxidation of reducing equivalent generated during glycolysis, TAO is expected as a candidate of target for chemotherapy against trypanosomes. It is also reported that TAO is highly homologous to alternative oxidase of mitochondria of plants, but biochemical analysis has not been carried out due to difficulty of purification. Therefore, we made overexpression of recombinant TAO (rTAO) in *E.coli* for the purpose of characterization of TAO, and turning ascofuranone to clinical use.

We established the condition on which about 80 percent of membrane fraction from large-scale culture of *E.coli* was rTAO. And, examination of solubilizing rTAO with various detergents revealed that digitonin is the most suitable detergent to solubilize rTAO. Digitonin-extaracted rTAO was purified by nickel column chromatography and enzyme assay of purified rTAO indicated that ascofuranone inhibited ubiquinol oxidase activity of rTAO competitively with ubiquinol, suggesting that aromatic part of ascofuranone is recognized by ubiquinol binding site.

## B－2) DIHYDROOROTATE DEHYDROGENASE CATALYZING SOLUBLE FUMARATE REDUCTASE IN *TRYPANOSOMA CRUZI*

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Succinate production resulting in reduction of fumarate is one of the essential processes controlling redox homeostasis for many organisms living under anaerobic conditions. However, *Trypanosoma cruzi*, a parasitic protozoa causing Chargas'disease, excretes a considerable amount of succinate even they uses TCA cycle and aerobic respiratory chain. Thus, it has been believed that unknown metabolic pathways are involved in the succinate production of the parasite. Present study shows the first evidence to characterize soluble fumarate reductase (FRD) of T.cruzi at the molecular level, which has been considered to be a key enzyme to understand the unknown pathways for the succinate production.

We presented here that *T.cruzi* soluble methylviologen (MV) -FRD consists at least 2 kinds of protein. One of the FRDs is attributable to dihydroorotate dehydrogenase (DHOD), catalyzing oxidation of dihydroorotate (DHO) to form orotate, a 4th enzyme of the pyrimidine de novo biosynthesis pathway. This indicates that not only *T.cruzi* DHOD functions as a part of the pyrimidine *de novo* bio-

synthesis pathway but also the enzyme may play an important role in fumarate/succinate metabolism. Using recombinant T.cruzi DHOD that was expressed in *E.coli*, it was demonstrated that the DHOD is biochemically belonging to family 1A enzymes that is soluble, homodimeric protein containing FMN. *T.cruzi* has unusual FRDs system in addition of aerobic respiratory chain. Further characterization FRDs of *T.cruzi* would provide us the complete view of the parasitic metabolism.

## B－3) COMPARATIVE ANALYSIS OF cDNA EXPRESSION PROFILES BETWEEN BLOODSTREAM AND PROCYCLIC FORMS OF *TRYPANOSOMA B.BRUCEI* USING FLUORESCENT DIFFERENTIAL DISPLY METHOD

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There are 200,000-300,000 Africa narcolepsy patients who are newly infected by Africa *Trypanosoma* every year. In addition, many domestic animals die from *Trypanosoma* infection. The *Trypanosoma* protozoa spread between the mammal host and the tsetse fly vector in its lifecycle. In order to control *Trypanosoma* protozoa in the future, we analyzed geneexpression profiles between blood stream and procyclic (insect) forms of *Trypanosoma brucei brucei* using the method of fluorescence differential display.

Briefly, total RNA of long slender, short stumpy and procyclic forms were reverse-transcribed. PCR was performed with combinations of 10-mer arbitrary primer and rhodamine CT16A, G, and C primers. After electrophoresis, differentially expressed cDNA bands were detected with fluorescent image analyzer.

As a result, about 10,000 cDNA (120 primer combinations) were surveyed, and a total of 39 differentially regulated cDNA bands were identified. One markedly differential expressed cDNA, which was upregulated in procyclic form, was selected and sequenced. No high similarity was found for this cDNA fragment, with Genbank database search. Thus, 5'RACE is now under way, for cloning of the full-length cDNA.

## B－4) PRELIMINARY ANALYSIS OF GENES EXPRESSED DURING *LEISHMANIA* PARATITES INFECTION USING cDNA MICROARRAY

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Purpose; The *Leishmania* is the causitive agent of leishmaniasis. It multiplies as the extracellular promastigotes and lives and replicates intracellularly in the mammalian macrophages. Macrophages play an important role agansit invasive microorganisms and viruses. It is very interesting why *Leishmania* parasite inhabits in the phagolysomes. On the other hand, cDNA microarray allows the simultanous parallel expression analysis of many genes. We report analysis data of gene expression during *Leishmania* parasites infection using cDNA microarray.

Materials and method: Total RNA were extracted from macrophage derived Balb/c mouse (J774-A1) and *Leishmania Leishmania major* (MHOM/SU/73/5ASKH) infected macrophages for 48 hrs culture. Total RNA derived *Leishmania*-infected macrophages labeled Cy5 (red) and control RNA labeled Cy3 (green). Two probe are combied,

then hybridized at 64℃ to microarray slide. Raw scanning image were processed using microarray image analysis software.

Results and discussion: Comparison between non-infected macrophages and *Leishmania*-infected macrophages verified the involvement of many genes. About one hundred genes were found to be up-regukated in *Leishmania*-infected macrophage. *Leishmania* afected gene expression of diverse range of cellular pathway and function, including cell adhesion, cytokine, farnesylation, metabolism, RNA handling, signaling, transcripition, transport factor and EST.

## B－5) DEFECT OF *LEISHMANIA* ANTIGEN-SPECIFIC INTERFERON-G PRODUCTION IN A PATIENT WITH DIFFUSED CUTANEOUS LEISHMANIASIS IN ECUADOR

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Cutaneous leishmaniasis is clinically divided into classical localized and diffused types. Clinical manifestations of diffused cutaneous leishmaniasis (DCL) are many nodules and papules on the whole body, but no ulcer. Those of localized cutaneous leishmaniasis (LCL) are one or several nodules, papules and ulcers, of which margin elevates like bank. DCL was actually not as rare as it was considered before. Many cases were recognized in the New world, especially in Brazil and Venezuela. DCL Patients shows humoral immunity against leishmania antigens, but not cellular immunity and delayed type hypersensitivity. To clarify leishmanian antigen-specific immune response and pathological characteristic of a patient with diffused cutaneous leishmaniasis.

The study was performed in Ecuador. A DCL patient caused by *L.* (*Leishmania*) *mexicana*, 6 patients with active LCL, 6 patients healed from LCL, 6 healthy doners who have been lived in urban area in Ecuador and were naïve to *Leishmania* infection were examined for leishmanian antigens-specific immune response. PBMCs prepared from above people were cultured with or without leishmanian antigen, PPD and ConA for 72 hours, and culture supernatants were collected and transferred to Japan. The concentrations of IFN-g and IL-4 in the supernatants were measured by ELISA.

PBMCs prepared from patients with active LCL and those healed from LCL produced enough amount of IFN-g in response to leishmanian antigen, PPD and ConA. Although PBMCs prepared from a patient with DCL also produced enough amount of IFN-g in response to PPD and ConA, but not in response to leishmanian antigen. Six healthy doners who have been lived in urban area in Ecuador also produced enough amount of IFN-g in response to PPD and ConA, but not to leishmanian antigen. IL-4 was not detected in any culture supernatants. Immune system of a DCL patient was shown to be in anergy or deviation to Th 2 against leishmanian antigen.

## B－6) CLINICAL SURVEY OF CUTANEOUS LEISHMANIASIS IN ECUADOR LAST 10 YEARS (1991～2000)

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We analyzed clinical and epidemiological data on cutaneous changes of leishmaniasis recorded from 1991 to 2000 in Ecuador. The study areas were included Provinces of Manabi, Los Rios, Bolivas, Pichincha, Azuay, and Esmeraldas located at the Pacific coast and the slope of Andean mountains. A total of 570 cutaneous leishmaniasis patients, 324 males and 246 females, living in Ecuador were examined for this study. Each patient was thoroughly examined clinically and parasitologically. The mean age of the patients was 19.82 years (±0.89 s.e.) in males, 17.45 years (±1.00s.e.) in females and 18.80 years (±0.67s.e.) in total. Patients less than 20 year-old occupied more than half of all the patients examined. The total mean onset of cutaneous lesions was 6.17 months (±0.41s.e.); 5.58 months (±0.39s.e.) in males, 6.96 months (±0.56s.e.) in females. 32 cases (6.5%) in total showed the duration period of lesions more than 13 months. The popular types of lesions included ulcer, nodule, erythematous plaque and papule. The most popular was ulcer formation. The patients with ulcer occupied more than 50% of the total. More than half of the lesions were located on the face and the extremities. Almost half of the lesions were solitary. The cases with more than ten multiple lesions were seen in a total of 16 patients. Ultrastructural observations showed poor parasitophorous vacuole (PV) formation in the skin specimens in this study. It is needed to examine whethet PV formation should be related to the duration of the diseases. Cutaneous leishmaniasis in Ecuador may differ from the other areas because of the defference of the parasite spesies, various behaviours of the vectors and the different life style of the inhabitants. Therefore, medical doctors assigned to the treatments for cutaneous leishmaniasis in Ecuador have not only medical knowledge but also entomological, ecological, environmental and anthropological knowledge.

## B－7) FLAGELLIN FROM BURKHOLDERIA PSEUDOMALLEI STIMULATES PULMONARY EPITHELIAL CELLS TO INDUCE IL-8 PRODUCTION

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The causative agent of melioidosis, Burkholderia pseudomallei (Bp) is one of the most important causes of mortality of septicemia and acute fulminant pneumonia in Northeastern part of Thailand. Increased plasma levels of IL-8 is associated with a high mortality in cases of septicemia, and is considered to be a better predictor of mortality. In this study, we examined the activity of IL-8 induction by pulmonary epithelial cell line (A549 cells) in response to the culture supernatant of B.p (CSBP), and determined which components were responsible for IL-8 induction by these cells.

We examined the IL-8 inducing capacity of CSBP of seven clinical isolates, and found potent activities of IL-8 induction in response to CSBP from these strains. A time- and concentration-dependent IL-8 production by A549 cells in response to CSBP (561 strain) was observed. Treatment with proteases and heating of CSBP significantly decreased IL-8 inducing activity. A gel filtration on chromatography of the CSBP on a Superose 12 column demonstrated one separate fractions showing IL-8 inducing activity. These were estimated to have molecular masses higher than 450 kDa.

Bacterial flagellin has been reported to stimulate NF-kb activation through toll-like receptor 5, which subsequently induces IL-8 production and other cytokines. We, therefore, doveloped an immunoblot assay for condensed filtrate of CSBP (MW cut off > 100 kDa) employing anti-flagellin of B.p antibody. Anti-B.p flagellin recognaized

the band MW of 43 kDa which corresponding to the known MW of B.p flagellin. These data suggest that one of major IL-8 inducing factors in CSBP strain is flagellin of B.p. Flagellin of B.p may contribute to dense neutrophil accumulation in the airway of patients with pulmonary mellioidosis.

## B－8) CLINICAL AND MICROBIOLOGICAL CHARACTERISTICS OF RESPIRATORY INFECTIONS AMONG HIV-INFECTED PATIENTS IN NORTHERN THAILAND

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**Background**: Prevalence of HIV infection is increasing in Thailand. However, clinical and microbiological characteristics of respiratory infections among such patients is not clear at present.
**Methods**: We prospectively analyzed the types and characteristics of respiratory infections in 160 HIV-infected patients (161 episodes, 112 males and 48 females, mean age 33.3 years) who were admitted to Nakornping Hospital in northern Thailand.
**Result**: The mean peripheral blood CD4 lymphocyte count (CD4/CD8) in 105 patients was 60.0/mm$^3$ (0.1). The most common diagnoses included pneumonia in 82, *pneumocystis carinii* pneumonia-like interstitial pneumonia in 33, pulmonary mycobacteriosis in 28, pulmonary nocardiosis in 8 and lung abscess in 8. The most common organisms detectde in the sputum included *Haemophilus influenzae* (34), *Pseudomonas aeruginosa* (9), *Streptococcus pneumoniae* (9), *Staphylococcus aureus* (8), *Penicillium marneffei* (7) and *Rhodococcus equi* (6). Twenty-three cases (14.3%) had mixed infections. Severe fungal infection caused by *Penicillium marneffei* in 15, septicemia in 11, life-threatening meningitis in 4 (cryptococcal in 3 and tuberculous in 1) and were noted, resulting in 20 fatalities (12.4%).
**Conclusions**: Several types of organisms and respiratory infections exist in HIV-positive patients in northern Thailand. In such cases, early diagnosis and treatment are important for a satisfactory clinical outcome.

## B－9) *MYCOBATERIAL INFECTION IN THAI CHILDREN INFECTED WITH HUMAN IMMUNODEFICIENCY VIRUS IN THAILAND*

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More than ten per cent of chidren, who are born to mothers infected with human immunodeficiency virus-1 (HIV), are still infected with HIV in Thailand. Half of these HIV-infected children contract serious respiratory infections, including tuberculosis (TB) and other common bacterial infections. In the present study, we established a mycobacteriology laboratory in a children hospital in Thailand, and carried out the following TB diagnosis examination for specimens from 105 chilren suspected with tuberculosis infection, including 14 HIV-infected children; acid-

fast staining, OGAWA eggbased culture, the MIGT culture system (Becton Dickinson) which monitor levels of oxgen by fluorescence, PCR diagnosis (Apmricore, Roch Scientific System) for *M.tuberculosis*, as well as *M.avium* and *M. intracellulare*, and a strain-specific DNA-hybridazation-based diagnosis kit, DDH Mycobacteria (Kyokutoh). In 91 HIV-negative children, 7 specimens form 7 children among 81 specimens was positive for *M.tuberculosis*. No nontuberculous mycobacterium was not detected in these specimens. In 16 HIV-infected children, among 16 specimens, 4 specimens from 2 children was positive for *M.tuberculosis*, 7 specimens from 5 children was positive for nontuberculous mycobacteria, such as *M.gordonae, M.Szulgai, M.triviale*, *M.simia*, *M.chelonae*, *M.scrofulaceum* and *M.intracellulare*. Three specimens from 2 patients was positive for both *M.tuberculosis* and nontuberculous mycobacterium. These results indicates that HIV-infected children were infected with *M.tuberculosis* as well as nontuberculous mycobacteria.

## B－10) COLLABORATION OF MEDICAL TEAM AND FIELDEPIDEMIOLOGIST DURING THE OUTBREAK OF EBOLA HEMORRHAGIC FEVER IN UGANDA

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On October 16,2000,WHO confirmed that the outbreak of febrile disease in Gulu district of Uganda was caused by Ebola hemorrhagic fever. WHO immediately sent personnel and started necessary measures in collaboration with Ugandan Ministry of Health. At the same time, the WHO requested international support, and the United States, France, Italy and Doctors Without Frontiers dispatched the personnel. Japan provided the financial support in the initial phase. In addition, Japanese government decided to dispatch medical specialists and sent 2 doctors on October 30.

The WHO established teams for the following 5 activities:

1. Field Epidemiology 2. Patient Treatment 3. Diagnostic Laboratory 4. Public Education 5. Management and Support

In this region, the smallest social unit is a nucleus family, and these blood relatives gather and form a colony. The chracteristic of this type of society is that groups of cosely related are scattered in a community. In this society, infectious disease rapidly propagates in the community through family and colony. Therefore, early identification and isolation of infected is established became primary activities.

The mobile team identified potential infection from contact early by follow-up of infected, and they were also in charge of education for prophylaxis of the disease and discovering undetected cases through regional patrol.

In the previous outbreaks of Ebola viral hemorrhagic fever, hospital infection and consequent reintroduction to community became a problem. By practicing the barrier nursing technique, the medical team prevent hospital infection.

With the collaboration of the mobile team and the patient treatment team, the outbreak of Ebola viral hemorrhagic fever in Gulu district of Uganda ended in February 2001.

In the outbreak such as Ebola hemorrhagic fever, the social structure affects transmission because of the “infection by contact”. Therefore, in controlling these diseases, epidemiological considerations including social structure of community, habit and behavior have to be taken into account, and the collaboration between patient treatment and field epidemiology is essential.

## B－11) DETOXIFYING EFFECTS OF *ACHYRANTHES JAPONICA* EXTRACT AGAINST HABU VENOM

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*Achyranthes japonica* (Inokozuchi) has been believed as the herb to cure a venomous snake's bite in Kagoshima, Japan. We tried to examine biological effects of Inokozuchi for confirming this ancient tradition.

Leaves from fresh Inokozuchi were cut into pieces and ground into pastes. Pastes were filtered and filtrated liquid was centrifuged at 5,000 rpm for 20 minutes and 5℃. Supernatant was freeze-dried. Freeze-dried Inokozuchi powder was dissolved in 1/60 M PBS and added *Trimeresurus* (Habu) venom (Sigma). These were mixtured and left for one hour in a room temperature. Five doses of Habu venom -Inokozuchi mixture were injected to mice and rabbits and calculated anti-lethal (ED50) and anti-hemorrhagic reactions of Inokozuchi extract. Five doses of Habu venom-boiled (one minute) Inokozuchi mixture were also injected to rabbits and calculated anti-hemorrhagic reactions of Inokozuchi extract. Tail pinch methtod was used to evaluate an analgesic level in mice administered Habu venom or Habu venom-Inokozuchi mixture.

Survival rates showed that median lethal dose in the mice administered Habu venom was 4.6 mg/kg of test Habu venom (LD50) and that median lethal dose in the mice administered Habu venom-Inokozuchi mixture was 10.5 mg/kg of test Habu venom (ED50). The ratio of LD50 to ED50 suggests that Inokozuchi reduced lethal toxicity of Habu venom by half. Fifty micro grams of Inokozuchi decreased hemorrhagic reaction due to Habu venom by 72.3%, although Habu venom-boiled Inokozuchi mixture could not decreased hemorrhagic reaction due to Habu venom. Inokozuchi suppressed tail pinch response due to Habu venom by 60%. Local necrosis could not be suppressed by Inokozuchi.

Therefore, Inokozuchi was proved to be antivenom of Habu venom and that involved effective substances were heat-unstable as a protein. Anti-hemorrhagic reactions of Inokozuchi is presumed to be catabolism of toxic proteins owing to enzymes of Inokozuchi.

## B－12) THE USE OF TRAVEL VACCINES BY JAPANESE EXPATRIATES IN DEVELOPING COUNTRIES

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We conducted a survey to clarify the level of use of travel vaccines by Japanese expatriates in developing countries. In 1999, we distributed a questionnaire to Japanese adults (more than 3000) living in Asia (East, South East and South), the Middle East, Africa and Latin America. As a result, 2895 questionnaires were recovered and the data were analyzed.

The analysis showed that 1398 Japanese (48.3%) were inoculated with some type of travel vaccines before departure. Compared with a similar survey conducted in 1994 and 1998, the ratio of inoculations (42.7% and 45.6%, respectively) was significantly higher ($p < 0.05$). Japanese in Africa showed the highest percentage (90.0%), followed by South Asia (67.1%), Latin America (48.6%), South East Asia (43.6%), the Middle East (37.0%) and East Asia (34.5%). More than 20% in all areas were inoculated with hepatitis A and B vaccines and tetanus toxoid. The ratios were regionally high for rabies vaccine in Africa (44.1%) and South Asia (39.2%), yellow fever vaccine in Africa (84.7%), and Japanese encephalitis vaccine in South Asia (26.1%). In each area, the ratios of meningitis, typhoid fever and cholera vaccines were low. In tropical Africa, the ratios of hepatitis A and B vaccines were around 20% in 1994, which increased to above 40% in 1999. However, the ratio of cholera vaccine decreased from 40.7% in 1994 to 9.4% in 1999.

Recently, many Japanese expatriates have begun to use travel vaccines bifore departure. However, more than half still do not use vaccines at all. Therefore, it is vitally important to make Japanese expatriates more aware of the importance and effectiveness of travel vaccines.

## B－13) SURVEY ON PATIENTS WITH IMPORTED INFECTIOUS DISEASES ADMITTED TO THE DEPARTMENT OF INFECTIOUS DISEASES

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Two hundred ninety two patients were admitted to the Department of Infectious Diseases, Tokyo Metropolitan Bokutoh General Hospital from January 1,2000 to December 31,2000, and 30 of them were infected with some infectious diseases in foreign countries. Fifteen were Japanese men, 10 were Japanese women, and other 5 patients were foreign people. The presumptive place of contraction was India in 6 patients, Thailand in 4 patients, Philippines in 4 patients, Indonesia in 3 patients, Tanzania in 2 patients, China in 1 patient, Bangladesh in 1 patient, New Caledonia in 1 patient, Australia in 1 patient, Kenya in 1 patient, Nigeria in 1 patient, Uganda in 1 patient, South Africa in 1 patient, Mali or Senegal in 1 patient, Thailand or Nepal or India in 1 patient, and unknown in 1 patient. The number of patients of infectious enteritis was 13 (3 were bacillary dysentery, 2 were *Campylobacter* enteritis, 2 were enteropathogenic *E. coli* enteritis, 1 was paratyphoid fever, 1 was *Aeromonas* enterits, and 4 were enteritis due to unknown organism), malaria were 6 (3 were falciparum malaria, 1 was ovale malaria, 1 was falciparum malaria with ovale malaria, and 1 was falciparum malaria with malariae malaria), dengue fever was 3, AIDS was 3, pneumonia was 2, pyeronephritis was 1, bronchitis was 1, and entero viral meningitis was 1. Twenty nine patients became to be well and discharged, but 1 Ugandan man of AIDS was died.

## B－14) CURRENT STATUS OF IMPORTED MALARIA IN JAPAN SINCE APRIL, 1999

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Epidemiologic and therapeutic aspects of imported malaria in Japan have been examined since 1980 by sending questionnaires every year to more than 1,700 major hospitals.The new “Infectious Disease Prevention Law” came into force on April 1,1999. Imported malaria cases from that time till December 31,2000 were analyzed. 227 cases were reported, including 110 vivax malaria cases, 91 falciparum malaria cases, 11 ovale malaria cases, 2 quartan malaria cases, 5 mixed infections cases, and 8 cases due to unknown species. The rate of relapse of vivax malaria is 12.4% and the rate of recrudescence of falciparum malaria is 2.3%. Two fatal cases with falciparum malaria (2.3%) were reported. 179 cases (78.9%) were Japanese, and 48 cases were foreigners. 175 cases (77.1%) were male, and 52 cases were female. Concerning of possible place of contraction with vivax malaria, 28 cases (25.5%) were originated from Indonesia, 17 cases (15.5%) from India, 15 cases (13.6%) from Papua New Guinea. With falciparum malaria, 14 cases (15.9%) were originated from Tanzania or Kenya, 13 cases (14.8%) from Nigeria, 8 cases (9.1%) from Ghana. The purposes of travel for Japanese malaria cases were sightseeing (34.9%), working for NGOs (12.0%), working as Japan Oversea Cooperation Volunteers (7.8%), reporting (7.8%), and academic investigations (6.0%). Concerning the drugs chosen for falciaprum malaria, mefloquine alone occupied 38.6% and combination therapies occupied 45.5%. Concerning the drugs chosen for erythrocytic stages of Plasmodium vivax, chloroquine alone, quinine alone, Fansidar alone, and combination therapies occupied 67.6%, 12.0%, 8.3% and 6.5%, respectively. For prevention of relapse of vivax malaria, primaquine was used in 81.5% vivax malaria cases. These works were done by the Research Group for Development of Chemothrapeutic Agents against Tropical Parasitic Diseases (supported by the Japanese Ministry of Health and Welfare) and the Research Group for Clinical Evaluation of Orphan Drugs against Imported Tropical and Parasitic Diseases (supported by the Human Science Promotion Foundation).

## B－15) ANALYSIS OF MALARIA SURVEILLANCE DATA BASED ON INFECTIOUS DISEASE PREVENTION LAW IN JAPAN

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**Background**: Although indigenous malaria was eliminated in Japan in 1962, over 100 imported malaria cases are reported among Japanese travelers to and foreign visitors from endemic areas each year. According to the research of the orphan drugs for tropical diseases, diagnosis and treatment are often delayed, and 110-130 malaria cases were reported each year and a total of 14 deaths were reported from 1990-1999.

**Methods**: To identify the current problems and the public health importance of malaria in Japan and to describe the risk factors for malaria infection and fatal outcome, we analyzed data from the national malaria surveillance system for April 1999 to March 2001. To estimate the number of Japanese travelers and foreign visitors, we reviewed the annual statistical report of legal migrants.

**Results**: During the study period, 288 malaria cases were reported, 198 in Japanese and 90 in foreigners. Falciparum malaria (n=116) accouted for 60.3% of the cases from Sub-Saharan Africa and 24.1% of the cases from South-East Asia. Six patients (5 Japanese, 1 foreigner) with fatal cases were diagnosed a mean of 11.8 days after onset of symptoms (range, 5-26) and 110 non-fatal cases a mean of 6.7 days (range, 0-90) (p=0.32). Additional deaths that may have occurred after reporting are not included in the data, because the system does not request such follow-up information.

In 2000, 7 million (39.4%) out of 17.8 million Japanese travelers went abroad to malaria endemic countries/territories. Among them, 36 malaria cases were reported for Sub-Saharan Africa (174.0 cases/100,000 travelers) and 41 cases (1.5/100,000) for South-East Asia (relative risk, 114.5; $p < 0.001$).

**Conclusions/Public Health Impact**: Each year, over 100 imported malaria cases are reported among Japanese travelers. Japanese travelers, especially those visiting countries with a high risk for falciparum malaria, need to be educated about malaria prevention and the importance of early treatment. Japanese physicians should ask patients about their travel history and consider malaria in febrile patients returning from malaria endemic countries to avoid delay in diagnosis. The surveillance system should be improved to include the prognosis of cases to enable calculation of the case fatality rate and better assess risk factors for fatal outcomes.

## B－16) HOW COULD THE ISTM'S MAILING LIST TRAVELMED BE UTILIZED?

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With the ultimate goal of improving travelers' health, the International Society of Travel Medicine (ISTM) utilizes multiple electronic communication modalities. In order to disseminate and share information, and to improve mutual understandings these means include 1) a web page, 2) mass e-mail distribution through which important alerts and advisories are unilaterally delivered to all 2000 ISTM members, 3) TravelMed, a mailing list on which 450 voluntarily subscribed ISTM members from >30 countries are free to propose and interactively discuss travel medicine issues of their own choosing, and 4) GeoSentinel, a surveillance network of 25 sites worldwide reporting on travel-related illnesses and their geographic origins.

When we analyzed TravelMed postings from May to August 2001, the total number of issues discussed was 106, composed of 81 infectious disease topics and 25 noninfectious diseases topics related to the health of travelers. Among those infectious disease issues about half were on

vaccination and one fourth on malaria. The number of responses to each issue varied from zero to ≥21, with a peak at 2 responses.

Responses from host-country experts to inquiries about the disease situation in that location or on the availability of a given vaccine or on antimalarial in that country are extremely useful to subscribers. For some discussion topics, contradictory responses are posted. Fortunately in these cases, the respondents often present basic ideas, Personal experiences or the literature from which their opinions are derived. However, more active participation by leading experts in travel medicine should be further encouraged. Overall, TravelMed could be a very useful means of discussion and is expected to facilitate improvement of travel medicine practices.

We (M. K. & Y. U. ) have initiated the provision of Japanese summaries of TravelMed postings. At the moment, these summaries are disseminated on a mailing list operated by the Japan Overseas Health Administration Center in which 140 participants responsible for travelers' health are taking part. By recruiting additional coordinators, the entire contents of TravelMed could be summarized with less burden on each individual. Moreover, we intend to provide these summaries on an independent web page in the near future in order to facilitate the easy availability of the TravelMed archives.

## B－17) CEREBRAL METABOLIC CHANGES IN A PRIMATE MODEL OF SEVERE HUMAN MALARIA AS STUDIED BY POSITRON EMISSION TOMOGRAPHY

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Plasmodium falciparum infection can cause a severe neurological syndrome termed Cerebral Malaria (CM). CM is seen mainly in children and nonimmune tourists in tropical countries where infection with Plasmodium falciparum is endemic. The neuropathological hallmark of CM had been the sequestration of erythrocytes containing parasites in cerebral capillaries and venules. However, detailed mechanisms of altered consciousness during CM remains controversial. Although there have been several studies on hemodynamic changes in CM, direct measurements on the cerebral metabolic rate of glucose (CMRglc) have not yet been reported at all. In this study, local-CMRglu of Japanese monkeys (Macaca fuscata) infected with Plasmodium coatneyi, a primate model of severe human malaria with cerebral involvement, were studied by using high-resolution positron emission tomography (PET) using 2-[18F] fluoro-2-deoxy-D-glucose ([18F] FDG) for the first time. FDG-PET data for two cases suggest that CMRglc in frontal and temporal cortex was significantly reduced, while those of basal ganglia do not change. CMRglu reduction in cerebral cortex seem to be heterogeneous in severly infected monkey's brain. Reduction of full brain metabolism agree well with the "luxually perfusion" as indicated by previously reported hemodynamic changes in human CM. Neuropathological study of the same monkeys as studies by FDG-PET showed that preferential sequestration of parasitized red blood cells appeared in the cerebral microvasculature. However, petechial hemorrhage, focal endothelial cell damage or necrosis were not observed at all in the HE stained brain tissues. This implies that the reduction of CMRglc does not caused by damages in brain parenchyma. We think that the CMRglc reduction in CM may have a protective role to local hypoxia secondary to mechanical blockage of vessels by sequestration.

## B－18) GLUCOSE METABOLIC CHANGE OF SPLEEN IN MALARIA-INFECTED *MACACA FUSCATA*: CORRELATION OF FDG-PET RESULTS WITH PATHOLOGICAL FINDINGS

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Severe falciparum malaria shows a wide variation in its clinical presentation, such as hypoglycemia, cerebral malaria, renal failure, and pulmonary edema, in addition to the classical features of spikes of fever, hemolytic anemia, and hepatosplenomegaly. These serious clinical symptoms may precipitate various organs into alterations in glucose metabolism. Glucose metabolic changes in severe malaria, however, have not yet been investigated.

In this study, we applied whole body high-resolution positron emission tomography (PET) using 2-[18F] fluoro-2-deoxy-D-glucose ([18F] FDG]) in order to detect the effect of malarial infection in glucose metabolism, using Japanese monkey (*Macaca fuscata*) infected with *Plasmodeum coatneyi*, aprimate model of severe human malaria. The PET scans were performed for three infected and three uninfected animals. The standard uptake value (SUV) , a semiquantitative index of tissue uptake of FDG, was produced for each case. We also examined pathological changes of tissues of various organs.

The SUV of the spleen significantly increased after infection, up to twice to six times as much as that before infection. However, those of the liver, heart, and lung remained unchanged. Pathological examinations of the splenic tissues revealed marked expansion of white pulps, strong expression of MHC class II molecules on B cells and dendritic cells in lymphoid follicles, and enlargement of macrophages which had phagocytosed malarial pigments. These pathological findings suggest the activation of the inflammatory cells in the spleen. The activation of these cells probably caused the significant rise in splenic glucose metabolism.

## B－19) CEREBRAL RING HEMORRHAGES IN SQUIRREL MONKEYS INFECTED WITH *PLASMODIUM FALCIPARUM*

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The severity of neurological symptoms in malaria patients might be correlated with cerebral pathologic features in the brain. Ring hemorrhages are frequently found in the brains of patients with falciparum malaria. We report here the squirrel monkeys infected with *plasmodium falciparum* Indochina-I/CDC revealed cerebral ring hemorrhages similar to that observed in human malaria cases.

Bolivian squirrel monkeys (*Saimiri sciureus boliviensis*) were used in this study. A splenectomised monkey was inoculated intravenously with cryopreserved parasitized red blood cells (PRBC) infected with *P. falciparum* Indochina-I /CDC as a donor of fresh PRBC. On day 16, the blood was collected and followed by removing plasma, white blood cells and PRBC containing mature stage parasites. The fresh erythrocytes include with $10^9$ PRBC were inoculated to spleen-intact monkeys. Peripheral blood was collected and the parasite counts on Giemsa stained thin blood films were monitored every day. Clinical signs were also observed daily. Neurological signs were assessed in accordance with “Blantyre coma scale” presented by world health organization. Between 7 and 11 days after inoculation, autopsy or necropsy was done. Histopathological ex-

amination was performed on their cerebrum and cerebellum. PRBC were found on day 1 followed by abrupt development of parasite counts. Clinical signs included anorexia, inactivity, convulsions and lethargy occurred about 1 week after the inoculation. In several monkeys, coma scale reached at < 3 as the state of unrousable coma. In gross findings, petechiaes were seen on the cut surface of white matter in the cerebrum and the cerebellum. In microscopic findings, ball, ellipse or ring shaped hemorrhages were seen in the cerebral and cerebellal parenchyma. These hemorrhages were usually situated around the occluded vessels in the white matter of the cerebral tissue just below the cortex. In the cerebellum, hemorrhages were generally adjacent to the granular layer within the white matter. They were 200 ～500 $\mu$m size in the major axis. In ring hemorrhages, a perivascular necrotic zone separated the exravasated erythrocytes from blood vessels.

In summarry, bolivian squirrel monkeys inoculated with *P. falciparum* Indochina-I/CDC infected fresh erythrocytes caused cerebral disorder and hemorrhagic lesions which is typical of human cerebral malaria. Due to difficulty of obtaining fresh tissues from patients, mechanism for the occurrence and participation in the neurological symptoms remain obscure. Understanding of the mechanisms would greatly benefit from the existence of a model for cerebral malaria in an experimental host closely related to man. Bolivian squirrel monkey might be a good model for studying ring hemorrhages associated with human falciparum malaria.

## B－20) IDENTIFICATION OF MALARIAl ANTIGEN-MIMICKING PEPTIDES

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Malaria antigen-mimicking peptides were selected by biopanning using a pentadecamer random peptide displayed phage library and anti-malarial anti-sera from 5 *plasmodium falciparum*-infected patients. Seventy-five positive phage clones were selected by ELISA assay after 3rd round biopanning. From the sequence analysis, 8 positive phage clones were classified as patient IgG-binding peptides and 34 clones were classified as IgM-binding peptides. There were several consensus alignments in IgG-and IgM-binding peptides. Each consensus alignment-bearing phage clone showed the same or similar reactivity pattern in their classes when binding affinity of the 42 individual phage clone to 20 patient sera and 10 control sera were examined by ELISA, suggesting the consensus alignments are critical for binding to the antibodies. When mixed 4 represent peptides were used for ELISA, the mixed peptides reacted with 40% and 10% of the patient sera from the uncomplicated malaria and those from sever and complicated malaria, respectively. Whether the relationships between symptoms and the reactivity are presence or whether the antibodies against these peptides inhibit the malarial infection are under studying.

## B－21) CONSTRUCTION OF ANTI-*P. FALCIPARUM* /ANTI-CD3 BISPECIFIC scFv WITH ANTIPARASITICIDAL FUNCTION

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A novel bispecific single-chain antibody fragmernt (biscFv) has been constructed to address the possibility of a new malaria therapeutic drug development. The biscFv consists of two different single-chain antibody fragments linked by a flexible peptide linker $(Gly_4 - Ser)_3$. One specificity of the two Fv fragments is directed against MSP-1 molecule on the surface of *Plasmosium falciparum* merozoites and the other is directed against the CD3 antigen of human T cells; the former reacts with any strains of *P. falciparum* but doesn't have the growth inhibition activity. The construct was expressed in insect cells using recombinant baculovirus system. The antigen-binding properties of the biscFv are indistinguishable from those of the corresponding univalent single-chain antibody fragments. In the presence of human peripheral blood mononuclear cells (PBMC), the biscFv specifically induced IFN-γ and TNF-α in *P. falciparum in vitro* culture. These data suggest that the bridge formation between malaria parasites and T cells induces cytokine production at high level near the parasites, presumably resulting in activating NK cells and macrophages and then killing the parasites *in vivo*. Thus, the biscFv possesses highly selective malaria targeting properties and activates T cells simultaneously. Cell-mediated immune response induced by the biscFv could be great advantage for the passive immunotherapy against malaria infection.

## B－22) *PLASMODIUM VIVAX* TRANSMISSION-BLOCKING VACCINE: EFFICACY STUDY ON HUMAN ISOLATES

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Antibodies against *Plasmodium vivax* ookinete surface proteins expressed in yeast (yPvs25 and yPvs28) were produced in mice. Theses sera elicit potent transmission-blocking activity to *P. vivax* Sall strain. To test the efficacy of this vaccine candidate in the natural parasite populations, antisera against yeast expressed recombinant proteins, yPvs 25 and yPvs28, were produced in mice and rabbits. The efficacies of the antisera were tested with human isolates at malaria clinics in northwestern Thailand. Blood was collected from patients who came to the clinics and whose blood smears was positive for *P. vivax*. Antibodies from either mice or rabbits were diluted with normal human serum and added to patients' red blood cells. After mixing the infected blood was fed to *Anopheles dirus* using membrane-feeding method. Mouse serum immunized with PfMSP1-19expressed in yeast or pre-immunized rabbit serum was used as control for mosquito feeding. Mosquito infection was observed on day 7 post blood feeding. Ten to twenty mosquitoes were dissected for midguts and examined for oocyst development. Number of oocyst was recorded and compared among different treatments. For most human isolates, sera from mice immunized using alum as adjuvant showed complete inhibition of oocyst development. Sera from rabbits immunized with yPvs25 or yPvs28 + alum was less inhibitory than the mouse sera. Sera from rabbits immunized with yPvs25 or yPvs28 + Freund's adjuvant was more inhibitory, but still less than the mouse sera. The inhibitory activity correlated with the antibody titer measured by ELISA on recombinant protein.

## B－23) AN ATTEMPT TO IDENTIFY GENES CONTROLLING SUSCEPTIBILITY TO MOUSE SEVERE MALARIA

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*Plasmodium berghei* ANKA (PbA) infection in the C 57BL/6 (B6) strain of mice leads to the development of experimental severe malaria (ESM) and kills almost all of the animals 6-12 days after infection. In contrast, the DBA/2 (D2) strain of mice shows strong resistance to ECM, a majority of animals survive from ECM and dies 20 to 30 days after infection by other malaria-related complications, such as severe anemia and hyperparasitemia. So far, little is known about the genetic basis that accounts for the different susceptibility to ECM. In order to localize such genes on the mouse linkage map, we carried out a genome wide screening using an $F_2$ intercross population of susceptible (B6) and resistant (D2) strains.

Experimental infections were achieved by intraperitoneal inoculation of 1 x $10^5$ parasitized red blood cells. $F_2$ animals were phenotyped either as ESM susceptible or as ESM resistant based on survival until day 14 after infection.

Prior to the infection, genomic DNA were prepared from mice. A total of 93 informative microsatellite loci along all autosomes and X-chromosome were genotyped. Marker-Trait associations on each marker were assessed by chi-square tests.

We identified a genetic region on mid-chromosome 18 that showed statistically significant linkage to ESM susceptibility. We will continue to make effort to identify a true ESM susceptibility gene on this region by both positional and functional approach.

## B－24) BIOCHEMICAL ANALYSIS OF COMPLEXII FROM *PLASMODIUM FALCIPARUM* MITOCHONDRIA

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Since the energy metabolism of *Plasmodium falciparum* is quite different from that of mammalian host, the enzymes of energy transducing pathway of the parasite are promising targets of anti-malaria drugs. Energy requirement during the erythrocyte stage is provide by metabolizing glucose by anaerobic gycolysis. A functional TCA cycle is absent and ATP synthase has not been characterized in this stage mitochondria because α-ketoglutarate dehydrogenase doesn't function.

However, recent studies have shown the presence of functional respiratory chain in *Plasmodium* mitochondria, and the new anti-malarial drug, atovaquone, has been shown to inhibit the electron transfer of the $bc_1$ complex (complex III) and to collapse the mitochondrial membrane potential. The presence of an NADH-ubiquinone oxidoreductase (complex I) has been suggested by ability of NADH -linked substrates, such as glutamate, to stimulate ADP phosphorylation in trophozoites treated with digitonin. Evidently, mitochondrial electron transport system is essential for the survival of *plasmodium* in the host, although ATP is supplied mainly by glycolysis.

Complex II is one of the respiratory components and is the oxidization-reduction enzyme, which catalyzes conversion of succinate and fumarate. Complex II plays a key role in the energy metabolism of parasites such as *Ascaris suum*. The mitochondrial respiratory chain of *A. suum* changes dramatically during the life cycle. Complex II from the mitochondria of adult *A. suum* exhibits high quinol-fumarate reductase (QFR) activity and plays a key role in anaerobic metabolism as a terminal oxidase in the NADH-fumarate oxidoreductase system. In contrast, complex II from free-living third stage 1arvae (L3) shows much lower FRD activity, and functions as a succinate dehydrogenase (SDH) during aerobic respiration. If it considers that malaria parasite

growths under low oxygen, also in *P. falciparum*, it is likely that complex II functions as QFR and plays an important role in an energy metabolism system. Saraveratum *et al*. have purified complex II of *P. falciparum*, and we have cloned the genes of enzyme for the catalytic subunits, *SDHA* for flavoprotein (Fp) and *SDHB* for the iron-sulfur protein (Ip).

In the present study, using $N_2$ cavitation, we established a protocol to prepare the active mitochondria from *plasmodium falciparum*. This mitochondrial fraction showed SDH and SQR (Succinate-ubiquinone oxidoreductase) activity of complex II. The specific activity of SDH and SQR (5.64 nmol/min/mg and 4.74 nmol/min/mg, respectively, at 25℃) was higher than that of the homogenate from the saponin-treated *P. falciparum* (approx. 1 nmol/min/mg, at 37℃) reported by Suraveratum *et al*.. The malarial enzyme activity was inhibited by competitive inhibitor malonate, but a known inhibitor of mammalian mitochondrial SDH, 2-thenoyltrifluoroacetone (1mM), had no inhibitory effect on the malarial SDH activity. The chalcone showing antimalarial activity against the *in vitro* growth of *P. falciparum* had no inhibitory effect on the malarial SDH activity, although rat mitochondria was inhibited by Licochalcone A which inhibits the activity of fumarate reductase (FRD) of *Leishmania major* promastigote. These results suggest property of malarial complex II is different from that of the host mammalian enzyme. Thus. It could be an excellent target for antiprotozoal drugs.

## B－25) MOLECULAR CHARACTERIZATION OF A1-CYS PEROXIREDOXIN FROM THE HUMAN MALARIA PARASITE *PLASMODIUM FALCIPARUM*

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The human malaria parasite *Plasmodium falciparum* is exposed to reactive oxygen species that are generated from its endogenous metabolism (e. g. haemoglobin digestion) and during external attack by the host immune system. In addition, several anti-malarial drugs (e. g. chloroquine and artemisinin) can burden the parasite with substantial oxidative stress as a part of their anti-malarial effects. Peroxiredoxins are recently described family of antioxidants that are ubiquitous in living cells for metabolizing hydrogen peroxide and hydroxy1 radicals. We report here the molecular cloning and characterization of a 1-Cys peroxiredoxin from *P. falciparum*. Approximately 250 bp of the 5' region of peroxiredoxin cDNA was obtained from the *P. falciparum* expressed sequence tags (ESTs). The missing 3' region of the cDNA was amplified from the trophozoite mRNA by rapid amplification of cDNA ends (RACE) technique. The full-length cDNA of the parasite's peroxiredoxin (PfPrx-2) encoded a 220 amino acid polypeptide with a predicted molecular mass of 25.2 kDa. The presence of the PVCT consensus sequence flanking the conserved Cys47 confirmed that the PfPrx-2 was the 1-Cysperoxiredoxin. This is the first report of 1-Cys peroxiredoxin in protozoan parasites. The antioxidant activity of eliminating hydrogen peroxide by the recombinant PfPrx-2 protein was demonstrated using the ferrithiocyanate system. An elevated expression of the PfPrx-2 protein seen in cytoplasm of the trophozoites, the stage with active metabolism, suggests an association of the peroxiredoxin with the parasite's intracellular redox control.

## B－26) EFFECT OF MINOCYCLINE IN COMBINATION WITH ANTIMALARIA DRUG ON THE ELIMINATION OF *PLASMODIUM BERGHEI* AND CHLOROQUINE RESISTANT-*P. CHABAUDI* PARASITES IN MICE

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The emergence and spread of drug resistant *plasmodium falciparum* is a major problem for chemotherapy of malaria. Development of potent anitimalarial is important, but it is also necessary to investigate the possible use of antimicrobial agents with wide spectrum effect, which have already been accepted for clinical use in patients with various infectious diseases. A group of tetracyclines has been used for the treatment of drug-resistant falciparum malaria. Tetracylines were usually combined with qunine since these antibiotics require a longer time for an appearance of the inhibitory effect than other antimalarial drugs.

We previously reported that minocycoline (MC) showed more strong inhibitory activity against *in vitro* growth of drug-resistant *P. falciparum* parasites than conventional tetracycline and doxycycline. In the present study, we examined whether MC is useful for treatment of murine malaria when it is combined with artemether (AM) or chloroquien (CQ). Random bred ICR mice were intraperitoneally inoculated with $1\times10^5$ erythrocytes infected with *P. berghei* NK65 strain and the parasitemia was followed. Mice treated with MC alone at a does of up to 50 mg/kg for 7 days or AM alone at a dose of up to 2.5 mg/kg for 4 days were all dead. However, when mice were treated with MC (50 mg/kg) for 7 days in combination with AM (2.5 mg/kg) for 4 days, all mice were cured. In the next experiment, mice were intraperitoneally inoculated with $1\times10^6$ erythrocytes infected with *P. chabaudi* 3CQR strain, with showed CQ-resistant phenotype. Mice received CQ at a dose of 5 mg/kg for 4 days exhibited a similar level of parasitemia compared with that observed in untreated control mice, although an appearance of parasitemia was delayed in the treated mice. However, when mice were treated with CQ (5 mg/kg) for 4 days in combination with MC (50 mg/kg) for 7 day, parasites in the peripheral blood was completely eliminated. These findings suggest that minocycline should be further studied in clinical cases as an excellent candidate for chemotherapy of chloroquine-resistant malaria in combination combined with other antimalarials such as artemether and even with chloroquine.

## B－27) ANTI-MALARIAL EFFECTS OF DIPYRIDAMOLE *in vitro* and *in vivo*

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We examined anti-malarial effects of dipyridamole (DIP), a coronary vasodilator, *in vitro* and *in vivo*. *Plasmodium falciparum* Indochina 1 (IC-1), a chloroquine (CQ)-sensitive strain, and K-1, a multi-drug-resistant strain were grown in the presence of DIP or CQ. At 72hr culture time, DIP reduced 50% of parasitemias in IC-1 and K-1 cultures at concentrations of about 50 and 70 $\mu$M, respectively. In both cultures of IC-1 and K-1, CQ reduced the parasitemias in the presence of 10 or 50 $\mu$M DIP more effectively than in the absence of DIP. Parasitemias in IC-1 and K-1 cultures incubated with 500 $\mu$M DIP treated red blood cells (RBCs) were lower than those in cultures with DIP-non treated RBCs, suggesting that parasitemias decreased as the result of DIP binding to the RBC membrane. We examined effects of DIP on survival rates of C57BL/6N mice infected with *Plasmodium berghei* ANKA. Among ten infected mice, eight mice died at 10th day after infection, whereas two mice died among the infected mice administrated with DIP (1 mg/day/mouse) for three days via the tail vein. These results suggested that DIP had anti-malarial effects both *in vitro* and *in vivo*.

## B－28) DEVELOPMENT OF NEW ANTIMALARIAL DRUG-*IN VITRO* AND *IN VIVO* ANTIMALARIAL ACTIVITY OF ENDOPEROXIDES

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A number of medicines such as chloroquine and quinine are available for treatment of malaria, but the rapid developments of drug resistance are serious problems. Medicinal agents based on novel mechanisms of action are, therefore, required to overcome emergence of resistance and to control an ever-increasing number of epidemics caused by the malaria parasite.

We have confirmed the potential of 1, 2, 4, 5-tetraoxacycloalkanes as a new class of simple peroxide antimalarial drugs. In the preliminary study, some 1, 2, 4, 5-teraoxacycloalkanes have been found to be available for chloroquine-sensitive and -resistant *P. falciparum in vitro*. Especially, 1, 2, 6, 7-tetraoxaspiro [7.11] nonadecane (N-89) has a potent antimalarial activity for *P. falciparum* that the $IC_{50}$ value for FCR-3 strain is $2.5x\ 10^{-8}$ M, that for K1 strain is $2.6x\ 10^{-8}$ M and the selective toxicity (>300) is also remarkable. In addition, we investigated the *in vivo* antimalarial activity of N-89 by 4-day suppressive test. The $ED_{50}$ value of N-89 has shown 12 mg/kg/day (ip), which was required to cause 50% suppression of *P. berghei* in mice. In the experiments, the dose of 50 mg/kg/day of five of N-89 treated mice were all cured, in which malaria parasites were not observed in circulating blood streams after 60 days. Furthermore, no side effects such as diarrhea, body weight loss and mortality were observed during treatment with 1,600 mg/kg of N-89 (ip). As a control, mice treated with artemisinin (50 mg/kg/day, ip) were not cured and mice died due to *P. berghei* infection.

We evaluated the effect of oral treatments of N-89 (160 mg/kg) for three consecutive days beginning on the day of 1% parasitemia. Parasitemia declined have been found after 24 hours of drug administration, all mice were cured with no parasite reincrease or toxicity. Conversely, on similar treatment with artemisinin, malaria parasites were still observed in their blood streams, parasitemia become increased again. Our studies indicate that N-89 has a strong and continuous antimalarial activity.

To determine the mechanism of action, morphological changes of malaria parasites during the treatment of N-89 against *P. falciparum* were studied. After treatment with 100 times the $EC_{50}$ value of N-89, we have observed a condensation of parasite nuclei, shrinked cytosol, inhibition of early and middle trophozoite parasite and little hemozoin formation.

Our results suggest that endoperoxide, N-89, is a new antimalarial candidate against drug resistant malaria.

## B－29) A PUBLIC/PRIVATE PARTNERSHIP AMONG THE JAPANESE PHARMACEUTICAL INDUSTRY, THE GOVERNMENT AND WHO/TDR (JPMW) : A RESEARCH PROJECT FOR DISCOVERY OF NEW ANTIMALARIAL DRUGS IN JAPAN

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The **JPMW** project was established in October 1999 through a “public/private parthership” (PPP) among the **J**apanese **P**harmaceutical Companies, **M**inistry of Health, Labour and Welfare, Japan (MHLW), and **W**HO/TDR, in order to discover new type of antimalarial drugs in Japan by the Japanese efforts. This project is expected to run for five years. Research Center for Tropical Diseases, The Kitasato Institute, is identified as the screening center for the project under WHO contract as the Kitasato Screening Center (KSC). KSC is carrying out the evaluation of antimalarial activity for the compounds from the chemical libraries of 14 Japanese companies, along with some of its own natural products, both *in vitro* and *in vivo*, using the multi-drug resistant strain of *Plasmodium falciparum* and the rodent ma-

laria model, respectively. In many ways the JPMW project was nurtured in the context of such international initiatives as the Roll Back Malaria Initiative that was proposed by WHO in 1998 and the Hashimoto Initiative that was proposed by the Government of Japan in the same year.

The financial support to the KSC screening operations is granted from MHLW through WHO/TDR technical service agreements (Principal investigator: Ōmura, S. ) An entity called JPMW Coordination Center (JCC) is playing a key interface role as "the hub" in this project. By 31 May 2001, KSC has assayed 9,212 samples as *in vitro* screening. The results have been registered into WHO/TDR database, and individual companies were notified. Furthermore, KSC has assayed 32 compounds as *in vivo* screening by 31 May 2001. Out of these the KSC results showed 3 compounds that had an activity score 3 *in vivo*.

In this presentation, we report the background, systems and current status of this JPMW project. There is not many successful cases of public/private partnerships in the world. The fact that this project has been strategically supported by the International Affairs Division of MHLW and blessed by the Japan Pharmaceutical Manufacturers Association (JPMA) would mean that JPMW was recognized as a uniquely Japanese contribution to a major international technical cooperation scheme. Further, we think that it is possible for the development of the new anitimalarial drugs in Japan, going beyond the screening project. The Medicines for Malaria Venture (MMV) is taking an active note of the JPMW outcome.

## B－30) BOTH MOSQUITO-DERIVED XANTHURENIC ACID AND A HOST BLOOD-DERIVED FACTOR REGULATE GAMETOGENESIS OF *PLASMODIUM* IN THE MIDGUT OF THE MOSQUITO

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Gametogenesis of *Plasmodium in vitro* can be induced by the combined stimulus of a 5℃ fall in temperature and the presence of xanthurenic acid (XA). *In vitro* experiments showed that *P. gallinaceum* ($EC_{50}$ = 80nM) is much more sensitive to XA than *P. berghei* (9 $\mu$M), *P. yoelii* (8 $\mu$M), and *P. falciparum* (2 $\mu$M). However, in the mosquito vector we do not know whether the temperature shift and XA are the only gametocyte activating factors (GAF), nor do we know with certainty the true source (s) of XA in the mosquito blood meal. Previous studies indicate that XA is the only source of GAF in the mosquito. By defining, and then contrasting, the ability of an XA-deficient mutant of *Aedes aegypti*, with the wild type mosquito to support exflagellation and ookinete formation *in vivo*, we determined the roles of parasite-, mosquito- and host blood-derived GAF in the regulation of gametogenesis of *P. gallinaceum*. Removal of both host and vector sources of GAF totally inhibited both exflagellation and ookinete production, whilst the lack of either single source resulted in only a partial reduction of exflagellation and ookinete formation in the mosquito gut. Both sources can be effectively replaced/substituted by synthetic XA. This suggests 1) both mosquito- and vertebrate-derived factors act as GAF in the mosquito gut *in vivo*; 2) the parasite itself is unable to produce any significant GAF activity. Studies are underway to determine whether vertebrate-derived GAF is XA. This data may form the basis of further studies of the development of new methods of interrupting malarial transmission.

## B−31) STUDIES ON MALARIA MICRO-GAMETOCYTE EXFLAGELLATION INDUCING FACTOR IN THE SALIVARY GLAND OF *ANOPHELES STEPHENSI*

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During blood feeding, the salivary glands of *Anopheles stephensi* extremely reduces protein contents. We investigated midgut contents and prediuresis fluid of *An. stephensi*. In the midgut contents after full blood meal, we found some proteins reacted with rabbit anti-salivary gland serum, which did not react with the midgut contents of mosquitoes before feeding. This suggests that these proteins come from the salivary glands. After feeding we also found sporozoites in prediuresis fluid of *An. stephensi*, which had been infected with *plasmodium berghei*. These sporozoites may come from the salivary glands, because the density of sporozoites in prediuresis fluid correlated with the number of sporozoites in the salivary glands. These results indicate that *An. stephensi* mosquitoes draw back their own saliva into their midguts when they feed blood (Med Entomol Zool 51: 13-20, 2000).

We next found gamete activation factor (GAF), which induced exflagellation of *Plasmodium* microgametes, in the salivary glands of *An. stephensi*. The exflagellation was induced in a concentration-dependent manner in the supernatant of salivary gland's crude homogenate. Total amount of GAF in the salivary gland was higher than that in the midgut and the head. GAF in the salivary glands was heat stable and low molecule (<3,000 molecular weight). Analysis of the supernatant by capillary electrophoresis and UV absorbance profile showed that the salivary glands contained xanthurenic acid, which was previously identified as GAF in the head of *An. stephensi*. The exflagellation inducing activity in the salivary gland declined immediately after a blood meal, implying that GAF was in the saliva, and was delivered into the midgut together with the blood and induced exflagellation in the midgut (Biochem Biophys Res Commun 287: 859-864, 2001).

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