1999). Subsequently, malaria cases rapidly decreased, with the implementation of the National Malaria Eradication Service, which was established jointly by the South Korean government and the World Health Organization (WHO) in 1959 (Ministry of Health and Social Affairs, Republic of Korea, 1966). Consequently, after the 1970s, indigenous malaria cases were almost unheard of, though 2 such cases were reported in 1984 (Soh et al., 1985). During the same period a substantial number of imported malaria cases was reported (Chai, 2002).

However, indigenous vivax malaria reemerged in 1993; a South Korean soldier working at the western edge of the demilitarized zone (DMZ; the border between South and North Korea) in Kyonggi-do (Province), was confirmed to have contracted *P. vivax* malaria (Chai et al., 1994). Thereafter, the number of malaria cases increased exponentially year by year, peaking in 2000 (Feighner et al., 1998; Chai, 1999; Lee et al., 2002; Park et al., 2003). North Korea, which stated that it was free of malaria from the 1970s, also started reporting cases in 1998 from the northern part of the DMZ bordering South Korea (Chol et al., 2005). Since then, the number of malaria cases in North Korea has increased dramatically and reached around 300,000 in 2001. Therefore, in 1999, the North Korean government developed a national malaria control program in cooperation with WHO, to reduce the malaria burden (Chol et al., 2005).

In South Korea, during the period 1993-1996, the outbreak area was confined to the northern part of Kyonggi-do and northwestern Kangwon-do, near the DMZ (Chai, 1999). However, after 1997, the outbreak area extended in an easterly direction to the northeastern region of Kangwon-do and in a southerly direction in Kyonggi-do (Lee et al., 2002; Park et al., 2003), and it was feared that this southward trend would continue. To cope with this risk, the present national malaria control program was launched in 1997 (Korea Center for Disease Control and Prevention, Republic of Korea, 2002). This program includes early case detection and treatment, chemoprophylaxis of soldiers, vector control, personal protection, and financial aids to North Korea for malaria control.

In addition to control activities, meteorological (temperature and rainfall) and entomological factors (mosquito density) may have significant impacts on malaria transmission. For instance, the incidences of malaria were related to local climatic variables in China (Bi et al., 2003) and Rwanda (Loevinsohn, 1994). However, in South Korea, no published data is available concerning the relations between temperature, rainfall, the population density of the vector mosquitoes, and the incidence of malaria.

The aim of the present paper is to briefly summarize vivax malaria outbreaks over the period 1993 to 2005 in South Korea, and to analyze the efficacies of the control activities implemented since 1997, and the impacts of meteorological and entomological factors on disease occurrence.

## MATERIALS AND METHODS

Malaria is designated an important communicable disease and case details must be reported immediately to the Ministry of Health and Welfare in South Korea. In the present study, all cases reported since the first reemergence of indigenous vivax malaria case in 1993 were subjected to analysis. Patients' occupations, i.e., civilians, soldiers on duty, and retired soldiers, were obtained from the Communicable Diseases Information System (http://dis.cdc.go.kr) and from the Communicable Diseases Monthly Reports issued during the study period by the Korea Center for Disease Control and Prevention (KCDC), Ministry of Health and Welfare, South Korea. Information about malaria prevalence in North Korea and financial support for malaria control was obtained from the KCDC, World Health Organization (WHO) (http://www.who.int) and the United Nations Office for the Co-ordination of Humanitarian Affairs (OCHA), Pyongyang, Democratic Peoples' Republic (DPR) of Korea (http://www.humanitarianinfo.org/dprk) and from the United Nations (UN) (http://www.reliefweb.int).

The annual geographic distributions of malaria cases in South Korea over 12 years were determined by grouping cases by city and province where patients were located when a diagnosis of malaria was

made. Information about the time required to make a diagnosis of malaria after the onset of symptoms, were obtained from the reports of patients admitted to local health centers and hospitals in Kangwon-do, South Korea.

Meteorological data, i.e., mean temperature and rainfall for the main transmission period (the 6 mo period from May to October), recorded at local weather stations in Cheolwon-gun, Kangwon-do, a malaria endemic area near the DMZ, were obtained from the Korean Meteorological Administration, South Korea.

The population densities of adult anopheline mosquitoes, over 90% of which is *Anopheles sinensis*, the main vector mosquito for vivax malaria in the Republic of Korea (Chai, 1999), were determined during the transmission period at one location in Cheolwon-gun, Kangwon-do, from 1993 to 2004 by two (WS Seok and YS Kim) of the authors. Adult anopheline mosquitoes emerged from the first week of May (1-10 mosquitoes/trap/night) and disappeared from the last week of October (0-14 mosquitoes/trap/night). A black light trap (Nozawa type, Shinyoung Korea Co., Seoul, Korea) was hung from a fence about 1.5 m above the ground in shed housing one cow. Black light traps were operated without additional attractants from 18:00 to 06:00 hr twice a week during the study period. All captured mosquitoes were transported the following morning to the Kangwon Institute of Health and Environment, where they were identified, separated, and the number of anopheline mosquitoes was counted.

Mass chemoprophylaxis (1 chloroquine tablet; 300 mg base) has been administered by the Ministry of Defense to a total of 985,282 soldiers working around outbreak areas (northern parts of Kyonggi-do and Kangwon-do) weekly from 1997 to 2005. Chemoprophylaxis was also prescribed to a total of 12,189 US soldiers in South Korea during the period 1997-2000. Retiring Korean soldiers were advised to take primaquine 15 mg base daily for 14 days for chemoprophylaxis against the liver stage parasite at the time of their retirement.

Spearman's correlation analysis was used to examine correlations between the number of new malaria cases, year, climatic factors, i.e. annual mean temperature (°C) and rainfall (mm), and the annual mean number of mosquitoes trapped during May to October. The monthly mean number of anopheline mosquitoes, and the mean number of mosquitoes trapped weekly and annual totals were calculated from mean monthly numbers trapped during the 6 month transmission period. $P$ values of $< 0.05$ were regarded as statistically significant.

## RESULTS

During the past 13 years (1993-2005), at least 937,634 indigenous vivax malaria cases have been reported in the Korean peninsula (South Korea and North Korea) (Table 1). Based on available data between 1999 and 2004 in South and North Korea, the number of cases reported peaked in 2001 with 298,058 cases in the Korean peninsula. In South Korea, during the period 1993-2005, a total of 21,419 indigenous vivax malaria and 488 imported malaria cases were confirmed (Table 1). The indigenous malaria patients included 8,353 (39.0%) civilians and 13,066 (61.0%) soldiers, including 5,626 retired soldiers (26.3%) who had retired from military service for less than one year at disease onset. The number of reported cases peaked in 2000 with 8.9 cases per 100,000 of the South Korean population. Thereafter, the number of reported cases declined sharply by approximately 26-40% per annum to 1.8-2.9 cases per 100,000 of the population in 2004-2005 (Table 1).

The annual incidence rate (including retired soldiers discharged < 1 year prior to onset and soldiers on duty) peaked at 457.3 cases per 100,000 soldiers in 2000. The incidence decreased by more than 84% between 2000 and 2004, but then increased by 35% in 2005 (Table 1). The same trend, i.e., peak in 2000 followed by a sharp decline until 2004 and a rise in 2005, was observed both among serving and retired soldiers. Among civilians, the annual incidence rate peaked at 3.3-3.4 cases per 100,000 in 1999-2000, and then decreased to 0.9 in 2004, but increased again to 1.9 in 2005 (Table 1).

In 1999, total 95,960 malaria cases were reported in

**Table 1.** Vivax malaria cases reported annually among civilians and soldiers in South Korea and North Korea

| Group | Number of reported cases (Annual cumulative incidence per 100,000 population) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1993 | 1994 | 1995 | 1996 | 1997 | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | Total |
| South Korea | | | | | | | | | | | | | | |
| Civilians | 0 | 2 | 7 | 46 | 361 | 1,148 | 1,541 | 1,580 | 1,047 | 864 | 542 | 413 | 802 | 8,353 |
| | (0.0) | (0.0) | (0.0) | (0.1) | (0.8) | (2.5) | (3.3) | (3.4) | (2.3) | (1.9) | (1.2) | (0.9) | (1.9) | |
| Soldiers | | | | | | | | | | | | | | |
| Retired[a] | 0 | 1 | 12 | 25 | 207 | 1,127 | 996 | 1,273 | 756 | 472 | 279 | 159 | 319 | 5,626 |
| On duty | 1 | 18 | 88 | 287 | 1,155 | 1,655 | 1,085 | 1,288 | 685 | 430 | 282 | 236 | 230 | 7,440 |
| Subtotal | 1 | 19 | 100 | 312 | 1,362 | 2,782 | 2,081 | 2,561 | 1,441 | 902 | 561 | 395 | 549 | 13,066 |
| | (0.2) | (3.4) | (17.9) | (55.7) | (243.2) | (496.8) | (371.6) | (457.3) | (257.3) | (161.1) | (102.0) | (71.8) | (96.7) | |
| Total | 1 | 21 | 107 | 358 | 1,723 | 3,930 | 3,622 | 4,141 | 2,488 | 1,766 | 1,103 | 808 | 1,351 | 21,419 |
| | (0.0) | (0.0) | (0.2) | (0.7) | (3.6) | (8.3) | (7.7) | (8.9) | (5.4) | (3.9) | (2.4) | (1.8) | (2.9) | |
| North Korea[b] | ND[e] | ND | ND | ND | ND | ND | 95,960 | 204,428 | 295,570 | 240,339 | 46,251 | 33,677 | ND | 916,225 |
| | | | | | | | (432.3) | (920.8) | (1,331.4) | (1,082.6) | (208.3) | (151.7) | | |
| Total, indigenous cases | | | | | | | 99,582 | 208,569 | 298,058 | 242,105 | 47,354 | 34,485 | | 937,634 |
| US Army soldiers[c] | 0 | 1 | 0 | 14 | 34 | 47 | 53 | 42 | 29 | 41 | 23 | 15 | ND | 299 |
| Imported malaria[d] | ND | 6 | 30 | 41 | 40 | 63 | 53 | 41 | 43 | 44 | 61 | 37 | 29 | 488 |

[a]Retired soldiers, who were infected during military service in risk areas and developed febrile illness at home after discharge from the service.

[b]Data were obtained from webpages of World Health Organization (http://www.who.int), the United Nations Office for the Co-ordination of Humanitarian Affairs, Pyongyang, Democratic Peoples' Republic of Korea (http://www.humanitarianinfo.org/dprk), and from the United Nations (http://www.reliefweb.int).

[c]United States Army cases were diagnosed either in South Korea or after return to the United States.

[d]Imported malaria cases in South Korea, who were infected in Southeast Asia, Africa, Oceania, and in Central and South Americas.

[e]ND = no available data.

North Korea, but this increased explosively 3-folds between 1999 and 2001 (1,331.4 per 100,000 North Korean population), and after 2002 decreased sharply to 208.2 and 151.7 per 100,000 population in 2003 and 2004, respectively (Table 1).

The annual numbers of malaria cases reported by nationwide administrative districts (provinces and large cities) are given in Fig. 1, as sums of 2 years, from 1994-1995 to 2004-2005. Of the total 21,419 cases registered during the 12 year period, most (85.4%) developed febrile illness in northern provinces and cities near the DMZ (the highest risk areas), including 10,411 cases (48.6%) in Kyonggi-do, 3,083 (14.4%) in Kangwon-do, 2,710 (12.7%) in suburban Seoul, and 2,089 (9.8%) in suburban Incheon (Fig. 1). In Kyonggi-do, the most serious outbreak area, the peak incidence occurred in 1998 with 2,197 cases, and decreased gradually afterwards. However, in Kangwon-do, the second most serious outbreak area, the peak incidence of 825 new cases, occurred in 2000. Small numbers of cases were reported from various Provinces and Cities countrywide through 12 years, although these cases were predominantly among retired soldiers who had served in northern parts of Kyonggi-do or Kangwon-do about a year previously, thus indicating a long incubation period. The numbers of patients reported in other Provinces and Cities are shown in Fig. 1.

Meteorological data, i.e., annual mean temperature and rainfall, and mean mosquito population densities, during 1993-2004, were analyzed in terms of their relationships with the annual total numbers of malaria cases reported in Kangwon-do, South Korea (Table 2). Spearman's correlation analysis showed that the occurrence of malaria in high risk areas was correlat-

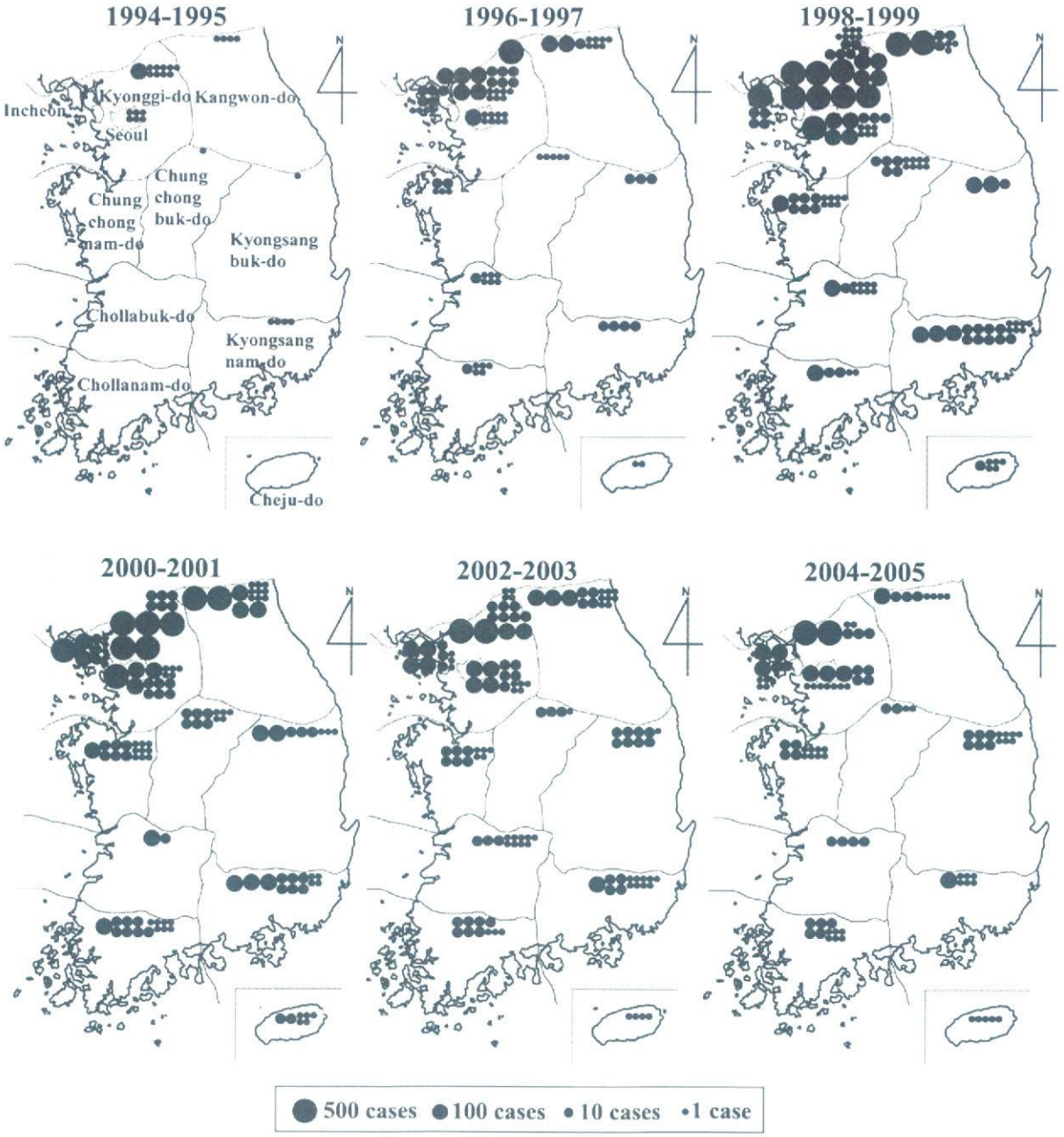


Fig. 1. Maps of South Korea, showing the numbers of indigenous vivax malaria cases reported by administrative districts (Provinces and Cities, including Incheon and Seoul) from 1994-1995 to 2004-2005. The figure represents the number of patients who developed febrile illness and were diagnosed in the district, but does not necessarily mean actual contraction of malaria in each district.

ed with the mosquito population, only with low significance ($P$ = 0.048), and no positive association was observed with temperature or rainfall (Table 2).

The time required for a diagnosis of malaria from the onset of febrile paroxysm has reduced year by year in most outbreak areas of Kangwon-do. For

**Table 2.** Mean annual temperatures, rainfalls, and anopheline mosquito population densities compared to annual malaria incidence rate in Kangwon-do, South Korea, from 1993 to 2004

| Item | Mean annual variables | | | | | | | | | | | | *P*-value[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1993 | 1994 | 1995 | 1996 | 1997 | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 | |
| Mean temperature (°C)[b] (May - Oct.) | 18.0 | 19.6 | 18.5 | 18.8 | 18.8 | 19.8 | 19.6 | 19.5 | 19.9 | 18.3 | 18.3 | 18.7 | 0.096 |
| Mean rainfall (mm) (May - Oct.) | 130.0 | 165.8 | 229.3 | 196.3 | 162.9 | 225.6 | 255.4 | 162.9 | 195.9 | 165.9 | 243.4 | 162.9 | 0.593 |
| Mean number of mosquitoes[c] (May - Oct.) | 604 | 799 | 779 | 290 | 550 | 1,293 | 1,801 | 1,574 | 890 | 1,194 | 1,282 | 1,299 | 0.048 |
| Annual number of patients (Malaria incidence; I)[d] | 0 (0.0) | 0 (0.0) | 4 (0.3) | 40 (2.6) | 177 (11.5) | 519 (33.4) | 514 (33.0) | 825 (53.1) | 544 (35.0) | 216 (14.0) | 132 (8.6) | 43 (2.8) | - |

[a]*P*-value: between the total number of patients and climatic variances (mean temperature and mean rainfall) and mean number of mosquitoes.
[b]Main transmission season in each year.
[c]Mean number of anopheline mosquitoes/cow/trap/night. Over 90% were *Anopheles sinensis*, the main vector mosquito.
[d]Incidence (I) per 100,000 population in Kangwon-do, South Korea. The correlation coefficient between I and mean temperature was 0.605, between I and rainfall 0.514, and between I and mosquito density 0.591.

example, 66 (44.0%) of 150 patients were diagnosed and treated within 6 days of symptom onset in 1999, but this increased to 61.7-73.6% during 2000-2002 (Table 3).

In 2001, the South and North Korean governments started to provide budgetary supports for facilitating malaria control programs in both countries. In the case of South Korea, 2 northern provinces (Kyonggi-do and Kangwon-do) and one city (Incheon), received budgetary supports for mosquito control from the KCDC, and this was followed by fiscal support from provincial and city health bureaus from 2001 to 2004. The total expenditures over this 4 year period in South Korea was 5,154,700 USD (Table 5). In North Korea, during the period 2001-2004, international supports for malaria control have been provided by WHO (for education and assistance for technician training), the International Federation of Red Cross and Red Crescent (IFRC) (anti-malarial drugs) and South Korea. The total amount of anti-malarial aid given to North Korea over this 4 year period was 3,150,650 USD (Table 5). In South and North Korea, during the same period (2001-2004), 8,305,350 USD were spent on malaria control. Items of supports provided by South Korea to North Korea included anti-malarial drugs (chloroquine and primaquine), mosquito control related materials and equipments (insecticide impregnated-bednets, personal protection fabrics, insecticides, and insecticide spraying equipment) and laboratory supplies for prompt diagnosis (microscopes and staining reagents) in local health centers and hospitals, and small amount of cash for the education of health personnel (Table 5).

## DISCUSSION

Our study demonstrated that the number of reemerging vivax malaria cases in South Korea increased exponentially during the years 1993-2000, but then decreased steadily until 2004 with a slight increase in 2005. This post 2000 decrease in malaria incidence was observed countrywide, and included high risk areas near the DMZ. Control programs were operated, including mass chemoprophylaxis, vector control, and financial aids to North Korea for malaria control, and are believed, at least in part, to have contributed to the reduction of malaria incidence.

Malaria transmission requires the combined presence of the *Plasmodium* parasite, the anopheline mosquito vector, and the human host. Both parasites and vectors are strongly affected by climate, for example, temperature determines parasite and vector develop-

ment, and rainfall provides the water required for vector breeding. In Rwanda and China, monthly mean temperature was found to play an important role in malaria transmission (Loevinsohn, 1994; Bi et al., 2003). However, with regard to rainfall levels, reports are contradictory; some studies have reported that rainfall is a key factor (Lindblade et al., 1999; Bi et al., 2003), whereas others have reported negative effects (Singh and Sharma, 2002). In a previous South Korean study, increases in temperature and precipitation were found to be correlated with seasonal vector mosquito population densities, and with the subsequent seasonal incidence of malaria (Lee et al., 2002). In this previous study, 2 climatic factors were compared with averaged data collected over a 30-year period, though no statistical analysis was performed (Lee et al., 2002).

In our study, low grade statistical significance ($P$ = 0.048) was observed in the correlations between anopheline mosquito densities and the annual malaria incidence during the 1993-2004 period in Kangwon-do, but none between the climatic variables and malaria incidence. Although mosquito densities during 1998-2001 could not be clearly correlated with malaria incidences, mosquito densities during these years were significantly ($P < 0.05$) higher than those before 1998 when malaria incidence was comparatively low (Table 2). Nevertheless, detailed ecological and epidemiological studies are needed to assess the true impact of climatic variables on malaria outbreaks in South Korea.

Regardless of the control strategy adopted, the early diagnosis and proper treatment of those infected is essential (Lee et al., 2003). In South Korea, the average duration between the onset of malarial fever and diagnosis at a health center or a hospital was 23.6 days in 1995, 9.5 days in 1997, and 8.0 days in 2000 (Park et al., 2003). Since 2000 about two-thirds of malaria patients have been more quickly diagnosed and treated, within 6 days, for example, in Kangwon-do (Table 3). Moreover, in 2004, the average detection time became as short as 3-4 days in highly endemic areas in Kangwon-do (data not shown), and it is speculated that early case detection substantially reduced the malaria transmission from patients to mosquitoes.

**Table 3.** Days required for confirmation of malaria diagnosis after the onset of symptoms among civilians and veterans in risk areas of Kangwon-do, South Korea, from 1999 to 2002

| Year | Number of cases (%) | | | | |
|---|---|---|---|---|---|
| | Days until diagnosis after the onset of febrile paroxysm | | | | |
| | 0-6 | 7-15 | 16-25 | > 26 | Total number of cases |
| 1999 | 66 (44.0) | 53 (35.3) | 18 (12.0) | 13 (8.7) | 150 |
| 2000 | 209 (73.6) | 66 (23.2) | 6 (2.1) | 3 (1.1) | 284 |
| 2001 | 138 (66.7) | 51 (24.6) | 12 (5.8) | 6 (2.9) | 207 |
| 2002 | 58 (61.7) | 21 (22.3) | 11 (11.7) | 4 (4.3) | 94 |

Mass chemoprophylaxis is another major contributor to the observed recent reduction in malaria cases. Before 1997, more than 80% of malaria cases occurred in northern parts of Kyonggi-do and Kangwon-do, the major outbreak areas (Chai, 1999; Park et al., 2003), and most patients were soldiers stationed near the DMZ. Therefore, mass chemoprophylaxis was administered to soldiers located in these endemic areas in 1997 and has continued ever since (Table 4). From 1997 to 2005, a total of 985,282 soldiers received chloroquine and primaquine prophylaxis. As a consequence, malaria incidence among soldiers on duty and retired soldiers decreased rapidly during 2001-2005. This prophylaxis program must have been largely responsible of the observed reduction in the malaria incidence.

However, it should be noted that the proportion of civilian cases among all malaria cases has increased from 38.2% in 2000 to 50.6% in 2004. This increase in the proportion of civilian cases suggests an increase in local transmission away from the DMZ (civilians usually live some distance from the DMZ). This increase in local transmission is also suggested by the fact that outbreak areas have expanded in southerly and easterly directions since 1998 (Chai, 1999; Park et al., 2003; Yeom et al., 2005).

It is also of note that a substantial number of cases

**Table 4.** Chemoprophylaxis of military soldiers of South Korea, US Army, and North Korea

| | Number of soldiers | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1997 | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | Total |
| ROK Army[a)] | 15,981 | 37,529 | 61,772 | 90,000 | 90,000 | 140,000 | 160,000 | 190,000 | 200,000 | 985,282 |
| US Army | 35 | 2,485 | 8,510 | 1,159 | ND[c)] | ND | ND | ND | ND | 12,189 |
| North Korea[b)] | 0 | 0 | 0 | 0 | 100,000 | 350,000 | 300,000 | 300,000 | ND | 1,050,000 |
| Total | 16,016 | 40,014 | 70,282 | 91,159 | 190,000 | 490,000 | 300,000 | 300,000 | 200,000 | 2,047,471 |

[a)]Republic of Korea Army.
[b)]Figures are based on the amount of anti-malarial drugs used for chemoprophylaxis and treatment supported by South Korea.
[c)]ND = no available data.

**Table 5.** Financial support for malaria control in South Korea and North Korea

| Group/Year | | Expenditures (in USD) | | | | |
|---|---|---|---|---|---|---|
| | | 2001 | 2002 | 2003 | 2004 | Total |
| South Korea | Mosquito control[a)] | 689,100 | 734,900 | 816,600 | 2,914,100 | 5,154,700 |
| North Korea[†] | Supported by South Korea | | | | | |
| | Anti-malarial drugs, mosquito control, etc.[b)] | 490,000 | 620,000 | 700,000 | 700,000 | 2,510,000 |
| | Education[c)] | 38,450 | 26,900 | 26,900 | 26,900 | 119,150 |
| | Supported by IFRC[d)] | | | | | |
| | Anti-malarial drugs, etc. | 21,000 | 21,000 | 158,000 | 321,500 | 521,500 |
| | Subtotal | 549,450 | 667,900 | 884,900 | 1,048,400 | 3,150,650 |
| Total | | 1,238,550 | 1,402,800 | 1,701,500 | 3,962,500 | 8,305,350 |

[a)]For insecticide purchase and equipments purchase for insecticide spraying in Kyonggi-do, Kangwon-do, and Incheon city, South Korea.
[b)]Anti-malarial drugs included chloroquine and primaquine (for treatment of 100,000-300,000 patients per year), and mosquito control included nsecticides like permethrin, devices for insecticide spraying, and insecticide-treated bed nets and clothes. Others included lancets, pH meters, staining reagents for blood smears and microscopes. Data are from World Health Organization, (WHO) and Korea Center for Disease Control and Prevention, South Korea.
[c)]For training laboratory technicians, entomologists, and health workers (total 70 persons per year) to help build a sustainable national ability to control malaria by WHO.
[d)]International Federation of Red Cross and Red Crescent Societies.

(more than 30% of all patients during the period 1998-2004) have been reported in Pusan, Taegu, Kyongsangbuk-do, and Kyongsangnam-do regions, which are considerably removed from major outbreak areas. Such cases may include retired soldiers, travelers to major outbreak areas, and locally infected civilians. In the case of retired soldiers living in these areas, the majority were probably infected while working in major outbreak areas, and developed febrile illness after a long incubation period of 5-13 mo (Chai, 1999), whereas travelers may have developed fever after a short (within 1 mo) or a long incubation period. It is unfortunate that no study has yet reported firm evidence of local malaria transmission in areas remote from the major outbreak areas.

There is no doubt that vivax malaria reemergence in South Korea was originally caused by infected mosquitoes originating from North Korea and the DMZ (Chai, 1999; Park et al., 2003). In this regard, it is worth mentioning that genotypes of circumsporozoite protein (Kho et al., 1999), merozoite surface protein (MSP)-1 (Zakeri et al., 2003), Duffy-binding protein

(Kho et al., 2001), apical membrane protein antigen-1 (Han et al., 2002), and MSP-3$\alpha$ (Han et al., 2004) of the reemerging vivax malaria in South Korea are similar to those found in the North Korean (NK) strain. Infected mosquitoes probably constantly migrate from North to South Korea (Cho et al., 2002), and we suggest that a large proportion of malaria cases in South Korea have resulted from this influx. Moreover, it is evident that the malaria situation in northern South Korea will be influenced by that in North Korea.

With regard to the malaria situation in North Korea, no data was available before 1997. However, recently some occurrence data has become available (Global Funds to Fight AIDS, Tuberculosis and Malaria, 2003; United Nations, 2003; United Nations Office for the Co-ordination of Humanitarian Affairs DPRK, 2003, 2004; World Health Organization, 2004; Chol et al., 2005). Indigenous cases have now been reported from 1997 (Chol et al., 2005), and nationwide patient numbers increased sharply prior to 2001, but then dramatically reduced to 2004. Several factors may have facilitated the increase in malaria cases during 1999-2001 in North Korea. Such factors may include changes in agricultural practices, such as, reduced use of pesticides and changes in rice field irrigation, intermittent big flooding, increased vector host densities, and inadequate health care delivery system.

However, a malaria control program was implemented in endemic areas of North Korea, in South and North Hwanghae-do (Provinces) during 2001-2003 by the National Program Office of WHO, in North Korea (Chol et al., 2005). It has been stated that the prevalence of malaria began to decline immediately after implementing this control program, and individual awareness regarding malaria increased rapidly. However, more precise data are required to better assess the situation of vivax malaria in North Korea.

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Short communication

# The *Plasmodium vivax* homolog of the ookinete adhesive micronemal protein, CTRP ☆

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Received 26 January 2006; received in revised form 14 April 2006; accepted 19 April 2006
Available online 5 July 2006

**Abstract**

The *Plasmodium* circumsporozoite protein/thrombospondin-related anonymous protein-related protein (CTRP) is expressed at the mosquito midgut ookinete stage and is considered to be a transmission-blocking vaccine candidate. CTRP is composed of multiple von Willebrand factor A (vWA) and thrombospondin type 1 domains in the extracellular portion of the molecule, and a short acidic cytoplasmic domain that interacts with the actomyosin machinery. As a means to predict functionally relevant domains within CTRP we determined the nucleotide sequences of CTRP from the *Plasmodium vivax* Sall and the *Plasmodium yoelii* 17XL strains and characterized the conservation of domain architectures and motifs across *Plasmodium* genera. Sequence alignments indicate that the CTRP 1st to 4th vWA domains exhibit greater conservation, and thereby are perhaps functionally more important than the 5th and 6th domains. This point should be considered for the development of a transmission-blocking vaccine that includes CTRP recombinant subunit. To complement previous cellular studies on CTRP, we further determined the expression and cellular localization of CTRP protein in *P. vivax* and *P. yoelii*.


*Keywords:* *Plasmodium vivax*; Ookinete; CTRP; von Willebrand factor A domain

Malaria parasites possess an apicomplexan-specific class of molecules, termed the TRAP/MIC2 family, which mediate adhesion onto host cell and tissue surfaces, gliding motility, and invasion of host cells. Members of this diverse family of transmembrane proteins are typically composed of one or more von Willebrand factor A (vWA) and thrombospondin type 1 (TSP1) domains in the extracellular portion of the molecule, and a short acidic cytoplasmic domain that interacts with the actomyosin machinery. Family members include the prototypic thrombospondin-related anonymous protein (TRAP) [1,2] that is expressed in *Plasmodium* sporozoite micronemes; the circumsporozoite protein/thrombospondin-related anonymous protein-related protein (CTRP) [3–7], localized to *Plasmodium* ookinete micronemes, and the *Toxoplasma gondii* tachyzoite micronemal protein, TgMIC2 [8]. TRAP/MIC2 family proteins are found across the apicomplexan clade, including NcMIC2 in *Neospora caninum*, Et100 in *Eimeria tenella*, and Em100 in *Eimeria maxima major* [9–11], and predicted homologs within the genome sequence of *Theileria annulata* (TA07755) and *Theileria parva* (TP04_0306). The *Cryptosporidium parvum* genome sequence lacks extracellular examples of the vWA domain and in this pathogen the predicted TRAP functional homolog (*Cp*TRAP-C1) is composed of Apple domains and TSP1 domains [12] (Fig. 1A).

*Abbreviations:* CTRP, circumsporozoite protein/thrombospondin-related anonymous protein-related protein; MIDAS, metal ion-dependent adhesion site; TSP1, thrombospondin type 1; vWA, von Willebrand factor A.

☆ Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL, and DDBJ databases under the accession numbers: AB247368–AB247370.

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doi:10.1016/j.parint.2006.04.003

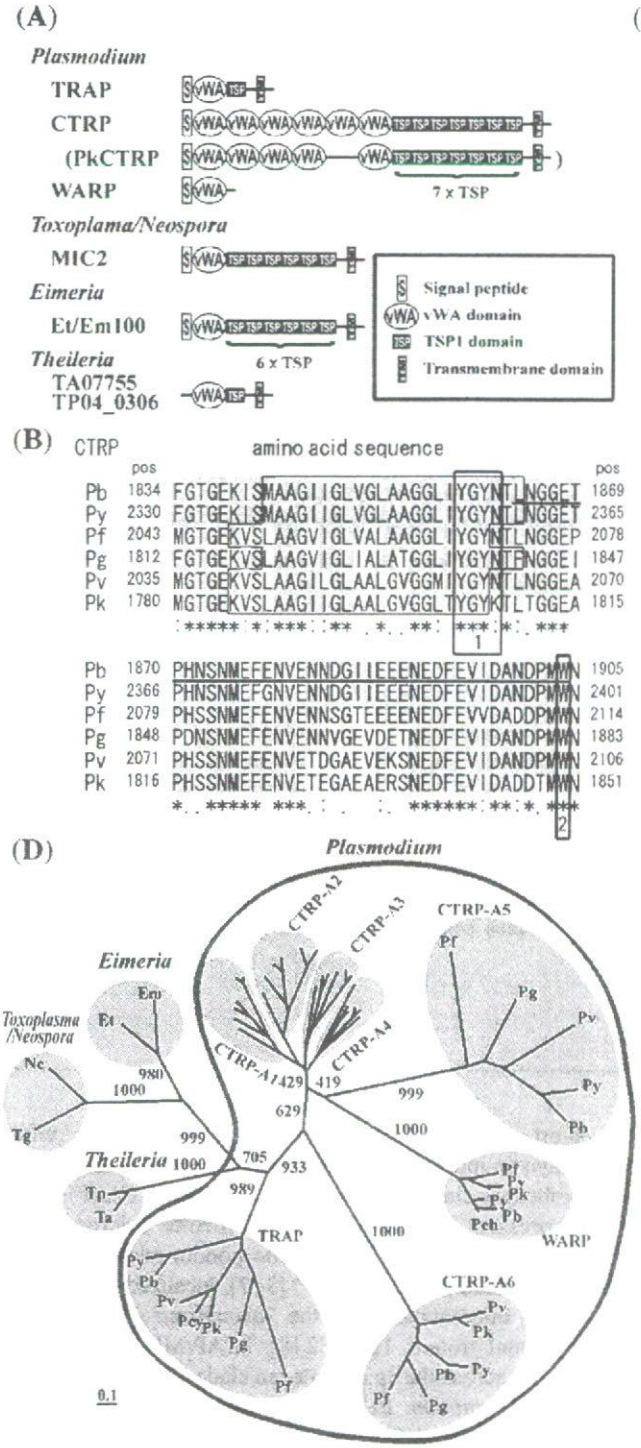


Fig. 1. (A) Schematic diagram of apicomplexan proteins containing vWA and TSP1 domains. Tg, *Toxoplasma gondii*; Et, *Eimeria tenella*; Em, *Eimeria maxima major*; TA, *Theileria annulata*; TP, *Theileria parva*. (B) Amino acid alignment of the transmembrane (boxed with thin line) and cytoplasmic regions of *Plasmodium* CTRP. The underlined region corresponds to that used to generate anti-*Pb*CTRP immune sera recognizing the cytoplasmic tail of *Pb*CTRP (amino acid position 1864–1904). Amino acids identical to those of *Pb*CTRP are shaded. Asterisks indicate the positions where amino acids are identical in all species, and similar amino acids are indicated with colons or periods under the alignments. The tyrosine-based motif involved in cellular trafficking (1) and the tryptophan residue that interacts with the motility actomyosin machinery (2) are boxed with thick lines. (C) Alignment of the MIDAS motif in the apicomplexan vWA domain superfamily. Numbers indicate intervening amino acids separating the three components of MIDAS (DxSxS, T and D, shaded). (D) Phylogenetic analysis of vWA domain of CTRP, TRAP, and WARP. Predicted amino acid sequences were aligned using the MUSCLE multiple sequence alignment program. Phylogenetic trees were constructed with deduced amino acid sequences by using neighbor joining method with Kimura's correction.

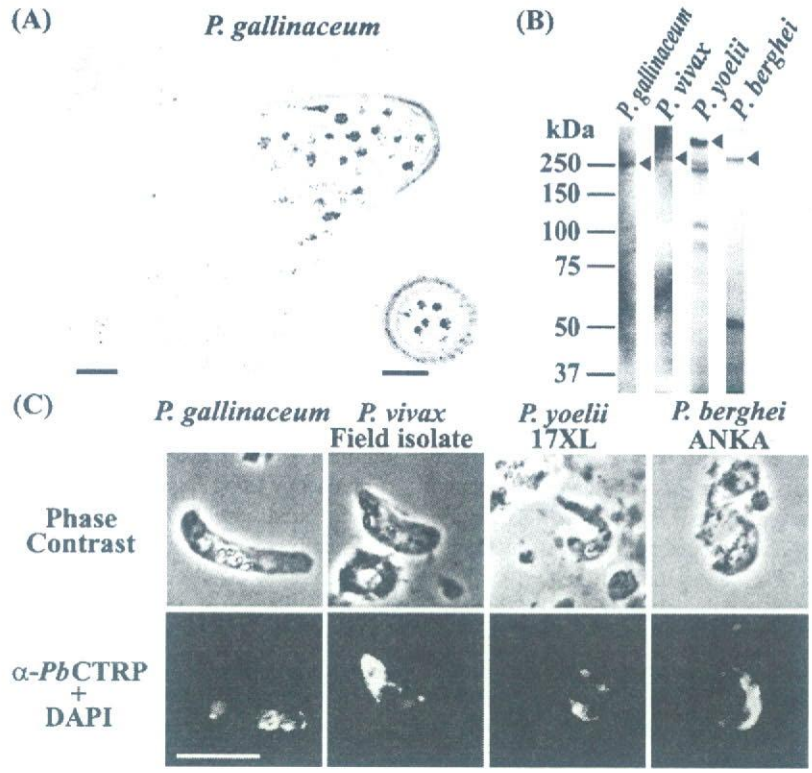


Fig. 2. (A) Immunoelectron microscopy of *P. gallinaceum* ookinetes using rabbit anti-*Pb*CTRP antibody. The immuno-gold particles associated with micronemes and the subpellicular region at the anterior portion. Bars indicate 0.5 μm. (B) Western immunoblot analysis of CTRP expression in *P. vivax* and *P. yoelii*. SDS-PAGE was performed with a 7.5% gel under reducing conditions. Proteins were transferred onto a PVDF membrane and reacted with rabbit anti-*Pb*CTRP antibody, followed by horseradish peroxidase-conjugated anti-rabbit IgG, then visualized by chemiluminescence (Immobilon Western Chemiluminescent HRP Substrate; Millipore, Billerica, MA). A band greater than 250 kDa was observed for *P. vivax* and *P. yoelii*. Anonymous bands smaller than 250 kDa were observed for *P. yoelii*, which may represent degraded *Py*CTRP protein. As a comparison, bands for *Pg*CTRP and *Pb*CTRP were visualized. An anonymous 50-kDa band was detected for *P. berghei*, which was also seen previously [6]. (C) Visualization of *Pv*CTRP and *Py*CTRP within the ookinete cytoplasm by confocal microscopy. Ookinete-stage parasites of *Plasmodium* parasites were stained with anti-*Pb*CTRP rabbit antibody, followed by FITC-conjugated anti-rabbit IgG. Phase contrast images are shown in the upper panels, and CTRP expression is seen in the cytoplasm of the parasites when FITC images were overlaid with DAPI-stained nuclei (lower panel). Bar indicates 10 μm.

The *Plasmodium* ookinete micronemal protein, CTRP, is essential for translocation of mosquito midgut ookinetes to the oocyst stage [4–7], and partial transmission-blocking activity was shown with antibodies recognizing CTRP in *Plasmodium gallinaceum* [13]. CTRP is thereby considered to be a transmission-blocking vaccine candidate. To understand the conservation of CTRP domain architectures and motifs across *Plasmodium* genera, we determined the nucleotide sequences of CTRP from the *Plasmodium vivax* SalI and the *Plasmodium yoelii* 17XL strains, for comparison with previously reported CTRP sequences and those retrieved from the genome sequence databases. To complement cellular studies on CTRP we determined the expression and cellular localization of CTRP protein in *P. vivax* and *P. yoelii*.

A partial DNA sequence of the putative *P. vivax* CTRP (*Pv*CTRP) gene was identified by a TBLASTN search of the *P. vivax* Gene Sequence Tag Project Website [14; accession number, AZ568294] using the amino acid sequence of *Pb*CTRP (AJ238798) as a query. The deduced amino acid sequence of this fragment had homology with the amino acid positions 1295–1464 of *Pb*CTRP, a region that spans the 6th vWA domain and the first TSP1 domain. Full-length *Pv*CTRP nucleotide sequence was obtained by anchored PCR gene walking as described [15], using four distinct *P. vivax* (SalI) genome DNA splinkerette libraries. PCR-amplified DNA fragments were inserted into the pGEM-T Easy plasmid (Promega, Madison, WI), and nucleotide sequences were determined (AB247369). The gene encoding the *P. yoelii* CTRP (*Py*CTRP) was isolated from the *P. yoelii* 17XL strain (AB247368), independently from the genome project, using a panel of degenerate oligonucleotides designed based upon alignments of the *pfctrp* (U34363) and *pbctrp* nucleotide sequences. Genomic DNA sequence for the *P. knowlesi* CTRP (*Pk*CTRP) was identified within the contig 4777 at the Sanger Centre website (http://www.sanger.ac.uk) following TBLASTN search using *Pv*CTRP amino acid sequence as a query. The reported genomic DNA sequence for the *P. gallinaceum* CTRP (*Pg*CTRP) was incomplete at the carboxy terminus [13; AB247370], and completion of the sequence was achieved using sequence identified within the *P. gallinaceum* genome database at the Sanger Centre website (2570384.c000412673.Contig1).

All CTRP genes possess predicted signal peptide sequences and single transmembrane regions, followed by a short (less than 50

amino acid), acidic cytoplasmic domain at the carboxy terminus (Fig. 1B). Similar to TRAP family members, a tyrosine residue is conserved in all species within the cytoplasmic domain near the transmembrane region; as well as a tryptophan residue at the penultimate amino acid position of the carboxy terminal (Fig. 1B). The tyrosine-based motif is proposed to target trafficking to the micronemes [16]; whereas the tryptophan residue is predicted to be involved in a hydrophobic interaction that is essential for interaction with the subpellicular actomyosin motility network [17]. The overall CTRP domain architecture, composed of six contiguous vWA domains followed by seven contiguous TSP1 domains, is conserved across species boundaries; with the exception that *Pk*CTRP lacks the 5th vWA domain and possesses only five vWA domains (Fig. 1A). Interdomain regions between adjacent vWA domains are easily discernable by characteristic insertions of low complexity proline-rich stretches of charged amino acids. The nucleotide sequence of *Py*CTRP of 17XL strain was identical to that of the 17X nonlethal strain that was used for the *P. yoelii* genome project. *Py*CTRP is notable for a greater than 420 amino acid long insert between the 5th and 6th vWA domains that is composed almost entirely of a repeat of glycine and asparagine residues. This insert is not shared with *Pb*CTRP, despite greater than 86% amino acid similarity throughout the protein.

The vWA domain metal ion-dependent adhesion site (MIDAS) motif [18] is relatively conserved in the first 4 vWA domains of CTRP, but has degenerated in the 5th and 6th vWA domains, and is additionally absent in the *Plasmodium* von Willebrand factor A domain-related protein (WARP) [13,19]. The MIDAS motif consists of a DxSxS consensus at approximately amino acid position 12–16 within each CTRP vWA domain, and threonine and aspartate residues conserved at approximately 89 and 121, respectively (Fig. 1C). This motif is well conserved across apicomplexan parasites and is thus likely to be important for domain function, such as contributing to the correct conformation for the receptor recognition by associating with the divalent metal ion. The MIDAS motif is degenerated in the WARP vWA domain, and because this molecule was found to be a multimer it does not appear that metal binding is essential for the multimerization.

To determine the evolutionary relationships of vWA domains in *Plasmodium* species, the amino acid sequences of this domain were aligned using the MUSCLE algorithm [20] with manual correction (Supplemental figure S1) and a phylogenetic tree was constructed by the neighbor joining method with Kimura's correction with bootstrap value of 1000. As out groups, apicomplexan proteins possessing both vWA and TSP1 domains were included: namely, TgMIC2, NcMIC2, Et100, Emt100, TA07755, and TP04_0306 (Fig. 1D). The amino acid diversities amongst the CTRP vWA domains are markedly different; for example, the 5th and 6th domains are more divergent compared to the first 4 domains as described [3], but also more diversified among *Plasmodium* species. Thus the functional constraints appear to be relaxed with respect to the 5th and 6th domains in comparison to the 1st through 4th domains, based on the following observations: 1) *Pk*CTRP lacks the 5th vWA domain, 2) the 5th and 6th domains are the most diverse amongst the *Plasmodium* species, and 3) the MIDAS motif is degenerated in these domains and the ability of receptor recognition is potentially lost. Alternatively, the first 4 vWA domains of CTRP might have evolved by concerted evolution with the gene conversion event between domains before *Plasmodium* speciation. Concerted evolution is known to reduce the diversity between homologous sequences.

The CTRP and WARP vWA domains form a single clade that is distinct from the TRAP vWA domain. This suggests that WARP, which contains a vWA domain but lacks TSP1 domains, originated from a CTRP vWA domain.

Because of the high amino acid sequence similarity (80 to 97.5%) between the cytoplasmic domains of CTRP (Fig. 1B), it was anticipated that cross-species reactivity would be observed for the described purified rabbit antibody to *Pb*CTRP cytoplasmic domain [6]. To evaluate this we performed immunoelectron microscopy using *P. gallinaceum* ookinetes. Indeed, gold particles were found associated with micronemes of mature ookinetes, especially localized at the periphery of each microneme and concentrated in the electron-dense subpellicular region just beneath the apical end (Fig. 2A). The gold particle associated electron-dense region appears to be circumferentially distributed around the apical pole and is consistent with the observed CTRP localization using antibodies specific to *Pg*CTRP [13]. Thus the cross-species reactivity of anti-*Pb*CTRP antibody was verified for *Pg*CTRP. The *Pb*CTRP antibody was further used to detect CTRP orthologs in *P. vivax* and *P. yoelii*. Western immunoblot analyses of ookinetes showed a band with the size slightly greater than 250 kDa for *P. vivax* and excessively greater than 250 kDa for *P. yoelii*. The protein sizes of *Pv*CTRP and *Py*CTRP were greater than those estimated from the amino acid sequences (229 kDa for *Pv*CTRP and 256 kDa for *Py*CTRP, after removing putative signal peptide sequences), which is frequently observed for the malaria proteins. Indeed, control *Pg*CTRP and *Pb*CTRP bands appeared around 250 kDa, which were estimated to be 210 kDa and 213 kDa, respectively (Fig. 2B). By confocal microscopy, anti-*Pb*CTRP antibody demonstrated a cytoplasmic patchy pattern in ookinetes of *P. vivax* and *P. yoelii* with deviated distribution, similar to the pattern for *Pb*CTRP and *Pg*CTRP (Fig. 2C).

In summary, we determined the *Pv*CTRP and *Py*CTRP nucleotide sequences and show protein expression in ookinete stages that is localized to the apical region. Sequence alignments suggest that the CTRP 1st to 4th vWA domains are functionally more important than the 5th and 6th domains. This point should be considered for the development of the recombinant subunits of a transmission-blocking vaccine based on CTRP.

## Acknowledgements

We thank Dr. M. Yuda for providing anti-*Pb*CTRP rabbit antibody. This work was supported in part by Grants-in-Aid for Scientific Research 16390125 and 16406009 (to T. T.), 15406015 (to M. T.), and 17590372 and 17406009 (to O. K.), and Scientific Research on Priority Areas 16017273 (to T. T.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. Preliminary sequence data of *P. knowlesi* and *P. gallinaceum* were obtained from the Sanger Institute website at http://www.sanger.ac.uk/.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.parint.2006.04.003.

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ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2007, p. 1071–1077 Vol. 51, No. 3
0066-4804/07/$08.00+0 doi:10.1128/AAC.01186-06


# Independent Evolution of Pyrimethamine Resistance in *Plasmodium falciparum* Isolates in Melanesia▽

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Received 22 September 2006/Returned for modification 1 December 2006/Accepted 18 December 2006

**Pyrimethamine resistance in *Plasmodium falciparum* has previously been shown to have emerged once in Southeast Asia, from where it spread to Africa. Pyrimethamine resistance in this parasite is known to be conferred by mutations in the gene encoding dihydrofolate reductase (*dhfr*). We have analyzed polymorphisms in *dhfr* as well as microsatellite haplotypes flanking this gene in a total of 285 isolates from different regions of Melanesia (Papua New Guinea, Vanuatu, and the Solomon Islands) and Southeast Asia (Thailand and Cambodia). Nearly all isolates (92%) in Melanesia were shown to carry a *dhfr* double mutation (CNRNI [underlining indicates the mutation]) at positions 50, 51, 59, 108, and 164, whereas 98% of Southeast Asian isolates were either triple (CIRNI) or quadruple (CIRNL) mutants. Microsatellite analysis revealed two distinct lineages of *dhfr* double mutants in Melanesia. One lineage had the same microsatellite haplotype as that previously reported for Southeast Asia and Africa, suggesting the spread of this allele to Melanesia from Southeast Asia. The other lineage had a unique, previously undescribed microsatellite haplotype, indicative of the de novo emergence of pyrimethamine resistance in Melanesia.**

Malaria is a major cause of morbidity and mortality in large areas of the tropical world. The antifolate drug sulfadoxine-pyrimethamine (SP) has been widely used to treat uncomplicated malaria, mainly as a monotherapy, but also in combination with other antimalarial drugs in most regions of endemicity for malaria in the world.

Pyrimethamine and sulfadoxine inhibit two separate enzymes in the folate synthesis pathway of *Plasmodium falciparum*: dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), respectively. Point mutations at amino acid positions 16, 50, 51, 59, 108, and 164 in the DHFR gene (*dhfr*) are the major causes of resistance to pyrimethamine (3, 17, 18, 24). The mutation at position 108 (Ser→Asn) appears to be an initial prerequisite for a significant (10-fold) increase in in vitro resistance (24). Additional mutations at other amino acid positions within the gene are associated with stepwise increases in resistance. Isolates harboring four mutations at positions 51, 59, 108, and 164 (CIRNL at positions 50, 51, 59, 108, and 164 [mutations are indicated by underlining]) show the highest pyrimethamine resistance so far described.

Various *dhfr* alleles have been observed in regions of endemicity (30). A *dhfr* triple mutant (CIRNI) represents the most common type in Africa and Southeast Asia, while the *dhfr* quadruple mutant (CIRNL) is observed predominantly in Thailand and some other regions in Southeast Asia where SP resistance is very high (1, 12, 30). Two distinct triple *dhfr* mutant genotypes (RICNI and CICNL) are prevalent in South America (2, 18). A five-amino-acid insertion after position 30, termed the Bolivia repeat, is also exclusive to South America, suggesting two unique and different evolutionary origins of pyrimethamine resistance in South America (2).

The migration of drug-resistant alleles can be traced by the analysis of microsatellite markers closely linked to the gene conferring resistance. Microsatellite analysis flanking *pfcrt* has revealed that chloroquine resistance evolved independently in at least four different regions: Southeast Asia, two regions in South America, and New Guinea (31). Meanwhile, all *dhfr* triple (CIRNI) and quadruple (CIRNL) mutants from Southeast countries displayed the same or nearly identical microsatellite haplotypes flanking *dhfr* (12). Strikingly, pyrimethamine-resistant isolates in Africa also harbored microsatellite haplotypes identical to those found in Southeast Asia (21), suggesting a single origin of pyrimethamine resistance in Southeast Asia, which subsequently spread to Africa. However, whether the Melanesian *dhfr* mutants originated in Southeast Asia or arose independently remains unclear.

In the present study, we determined *dhfr* and microsatellite haplotypes flanking the gene in *P. falciparum* isolates from Melanesia (Papua New Guinea, Vanuatu, and the Solomon Islands) and Southeast Asia (Thailand and Cambodia). Our

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▽ Published ahead of print on 8 January 2007.

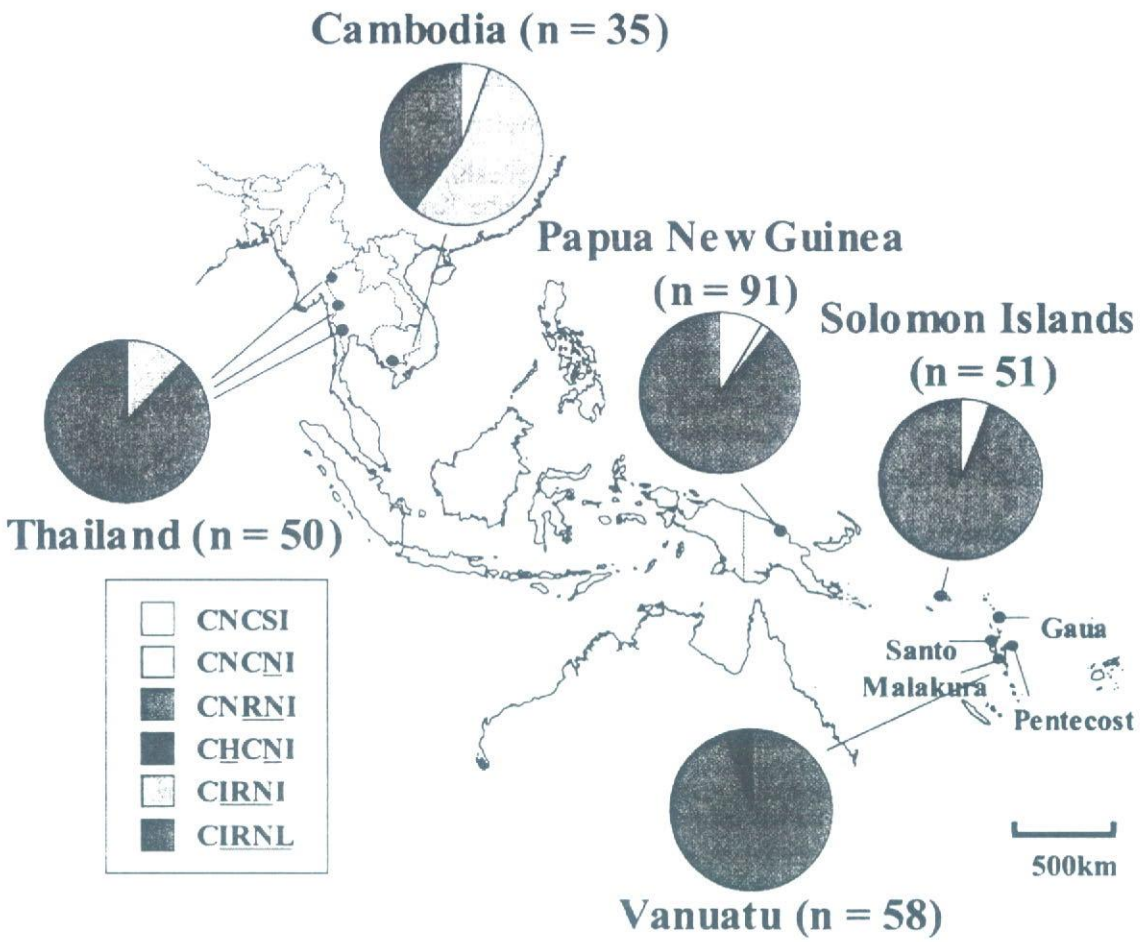


FIG. 1. Frequency of *dhfr* genotypes in *P. falciparum* isolates from Papua New Guinea, the Solomon Islands, Vanuatu, Cambodia, and Thailand.

results show two major lineages of pyrimethamine resistance in Melanesia. One has apparently originated in Melanesia, while the other originated in Southeast Asia and spread to Africa and Melanesia. This is the first unambiguous demonstration of the unique evolution of *P. falciparum* pyrimethamine resistance in Melanesia.

## MATERIALS AND METHODS

**Study site and patients.** Blood samples were obtained from *P. falciparum*-infected patients living in five countries where malaria is endemic: (i) Papua New Guinea, where isolates were from finger-prick blood samples taken during in vitro studies at a town clinic in Wewak, East Sepik Province, in November of both 2002 and 2003 (9, 10); (ii) Solomon Islands, where isolates from venous-blood samples were taken as part of a cross-sectional survey of three villages located in the northwestern part of Guadalcanal in 1995 and 1996 (23); (iii) Vanuatu, where isolates were from finger-prick blood samples obtained during a cross-sectional survey of rural villages and primary schools from February to March 1996 to 1998 in four islands, Gaua, Santo, Pentecost, and Malakula (22); (iv) Cambodia, where isolates were obtained from finger-prick blood samples taken during a cross-sectional survey of rural villages in Chumkiri, Kampot Province, in December 2004; (v) Thailand, where isolates were obtained from pretreatment venous blood samples taken during in vitro studies at a town clinic located at the western border of Tak, Kanchanaburi and Ratchaburi provinces, from 2001 to 2002.

**DNA preparation.** Finger-prick blood (75 μl) was spotted onto chromatography filter paper ET31CHR (Whatman Limited, Kent, United Kingdom). Venous blood was transferred into heparin-containing test tubes. Parasite DNA was purified using a QIAamp DNA blood mini kit (QIAGEN, Germany) according to the manufacturer's instructions with some modifications (22).

***dhfr* genotyping.** *dhfr* was amplified by PCR, and amplified products were directly sequenced with a BigDye Terminator 1.1 cycle sequencing kit in the ABI 377 DNA sequencer (GE Healthcare UK Ltd., Buckinghamshire, England) as previously reported (10, 19).

**Microsatellite haplotyping.** In order to determine the evolutionary history of pyrimethamine-resistant alleles of *dhfr*, we measured variation in the number of TA repeats at six microsatellite loci closely linked to the gene. These were located on chromosome 4, 0.1, 3.87, and 4.49 kb upstream and 0.52, 1.48, and 5.87 kb downstream of *dhfr*. In some cases, in order to estimate the limit of genetic hitchhiking, which is defined as a valley of reduced variation around *dhfr*, an additional six-microsatellite markers were analyzed at 7.55, 29.61, 57.68, and 363.33 kb upstream and 30.31 and 299.72 kb downstream of *dhfr*. Polymorphisms in these microsatellite markers were determined as previously described (12). Briefly, seminested PCR was performed using fluorescent end-labeled primers. Size variations in the amplified products were determined by electrophoresis on an ABI 377 sequencer and analyzed with GeneScan software (GE Healthcare UK Ltd.). Samples with two or more peaks at the same locus in the electropherogram were considered to be mixed infections and were excluded from further analysis.

Polymorphism between microsatellite markers is measured as variation in nucleotide length derived from various numbers of TA repeats. Microsatellite haplotypes harboring an association of bp 200-194-176-106-203-111 at microsatellite positions 4.49, 3.87, and 0.1 kb upstream and 0.52, 1.48, and 5.87 kb downstream of *dhfr* were designated "SEA" haplotypes, and those harboring an association of bp 220-202-156-100-205-111 were designated "Melanesia" haplotypes. Microsatellite haplotypes showing slight differences at one or two microsatellite markers from the SEA haplotype, e.g., at bp 204-200-176-106-203-111

TABLE 1. Microsatellite polymorphisms in 15 *P. falciparum* isolates with wild-type *dhfr* or single-mutant *dhfr*

| Isolate | Country[a] | Size (bp) of microsatellite marker at indicated position (kb) | | | | | |
|---|---|---|---|---|---|---|---|
| | | −4.49 | −3.87 | −0.1 | +0.52 | +1.48 | +5.87 |
| CNCSI | Cambodia | 198 | 206 | 156 | 94 | 203 | 105 |
| (*n* = 10) | Cambodia | 198 | 206 | 156 | 94 | 203 | 105 |
| | PNG | 202 | 196 | 156 | 94 | 203 | 121 |
| | PNG | 214 | 198 | 156 | 94 | 203 | 123 |
| | PNG | 202 | 192 | 156 | 96 | 203 | 115 |
| | PNG | 204 | 194 | 172 | 96 | 203 | 103 |
| | PNG | 204 | 194 | 172 | 92 | 203 | 103 |
| | PNG | 204 | 206 | 172 | 100 | 203 | 111 |
| | PNG | 202 | 192 | 176 | 96 | 203 | 115 |
| | PNG | 202 | 192 | 176 | 96 | 203 | 115 |
| CNCNI | Solomon | 210 | 194 | 172 | 96 | 203 | 113 |
| (*n* = 5) | Solomon | 204 | 208 | 176 | 94 | 203 | 120 |
| | Solomon | 204 | 208 | 176 | 94 | 203 | 120 |
| | PNG | 210 | 194 | 178 | 102 | 203 | 113 |
| | PNG | 210 | 194 | 178 | 102 | 203 | 113 |

[a] PNG, Papua New Guinea; Solomon, Solomon Islands.

(underlining indicates the differences), were considered SEA variation haplotypes. Similarly, haplotypes displaying minor variation at one or two markers from the Melanesia haplotype, e.g., bp 220-202-156-100-205-113, were considered Melanesia variation haplotypes. Isolates showing mixed *dhfr* genotypes and/or microsatellite haplotypes were excluded from analysis.

**Statistical analysis.** We calculated the expected heterozygosity ($h$) at each microsatellite locus as $h = [n/(n-1)][1 - \Sigma p_i^2]$, where $n$ is the number of infections sampled and $p_i^2$ is the frequency of the $i^{th}$ allele. The sampling variance of $h$ was calculated according to the following formula (23), a slight modification of the standard diploid variance (13), $[2/n(n-1)]\{2(n-2)[\Sigma pi^3 - (\Sigma pi^2)^2] + \Sigma pi^2 - (\Sigma pi^2)^2\}$. A $P$ value of $<0.05$ was considered statistically significant.

**Nucleotide sequence accession number.** The complete sequence of the allele identified has been submitted to the DDBJ and assigned accession number AB271908.

## RESULTS

***dhfr* genotypes.** Among a total of 314 samples, 29 (9%) had multiple *dhfr* alleles and/or were of mixed microsatellite haplotypes and so were excluded from this analysis. *dhfr* allele types and flanking microsatellite haplotypes were thus determined for 285 isolates (91 from Papua New Guinea, 51 from the Solomon Islands, 58 from Vanuatu, 35 from Cambodia, and 50 from Thailand). The frequencies of *dhfr* genotypes differed considerably between Southeast Asia and Melanesia (Fig. 1). In Southeast Asia, nearly all parasites (98%) carried either triple (CIRNI at positions 50, 51, 59, 108, and 164) or quadruple (CIRNL) mutations at *dhfr*. In Cambodia, triple and quadruple mutants were near equally prevalent. In Thailand, 85% of isolates were quadruple mutants. In Melanesia, nearly all isolates (92%) harbored a *dhfr* double mutation (CNRNI). The pyrimethamine-sensitive, wild-type allele (CNCSI) was found in only Papua New Guinea and at relatively low prevalence (8%). Neither the triple (CIRNI) nor the quadruple (CIRNL) mutant was found in Melanesia. A unique CHCNI allele was observed in three isolates from Gaua Island, Vanuatu.

**Polymorphism in microsatellite markers flanking *dhfr*.** The polymorphisms in six microsatellite markers flanking *dhfr* (−4.49 to 5.87 kb) from wild-type ($n$ = 10) or single-mutant ($n$ = 5) isolates are shown in Table 1. For those parasites carrying pyrimethamine-sensitive, wild-type alleles of *dhfr*, microsatellite markers were highly polymorphic. In contrast, *dhfr* double-mutant isolates ($n$ = 184) showed remarkably little diversity at all loci (Fig. 2). Similarly, nearly all triple ($n$ = 25) and quadruple ($n$ = 58) mutants displayed limited microsatellite polymorphism at each locus (Fig. 2). The expected heterozygosity ($h$) at each microsatellite marker is given in Table 2. In isolates carrying wild-type or single-mutant *dhfr* alleles, $h$ was high (0.60 to 0.89) at all six loci located between 4.49 kb upstream and 5.87 kb down-

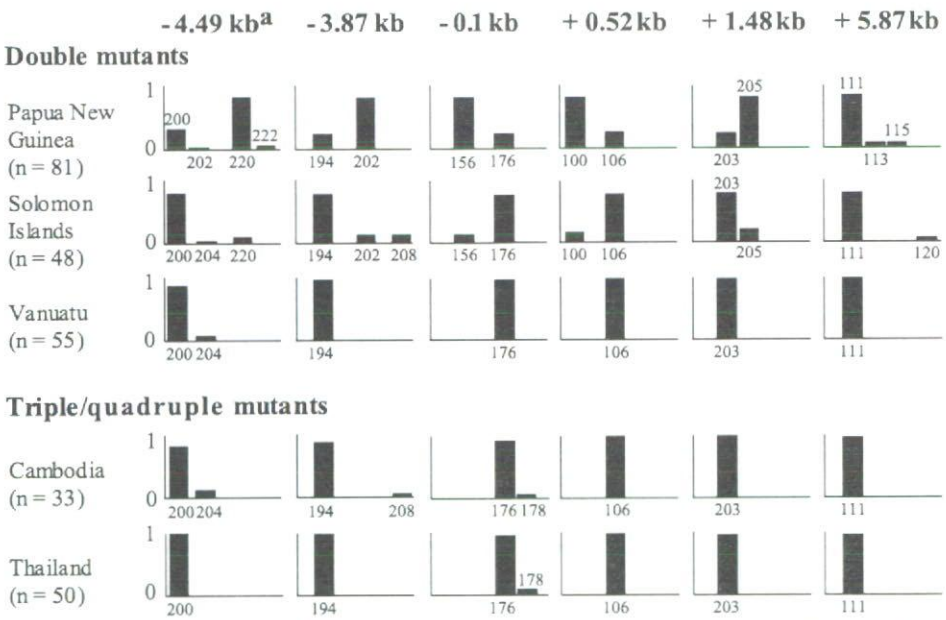


FIG. 2. Repeat length variations of six microsatellite markers flanking *dhfr* in *P. falciparum* isolates with *dhfr* double (CNRNI) and triple (CIRNI)/quadruple (CIRNL) mutants. $x$ axes, size (bp) of microsatellite markers. $y$ axes, frequency of microsatellite alleles. [a], location of microsatellite marker (distance from *dhfr*).

TABLE 2. Expected heterozygosity of microsatellite markers in *P. falciparum* isolates

| Isolate | No. of isolates | *h* of microsatellite marker at indicated position (kb) | | | | | | No. of haplotypes |
|---|---|---|---|---|---|---|---|---|
| | | −4.49 | −3.87 | −0.1 | +0.52 | +1.48 | +5.87 | |
| CNCSI | 10 | 0.78 | 0.84 | 0.69 | 0.71 | 0 | 0.89 | 8 |
| CNCNI | 5 | 0.60 | 0.60 | 0.80 | 0.80 | 0 | 0.60 | 5 |
| CNRNI | 184 | 0.54 | 0.49 | 0.48 | 0.49 | 0.48 | 0.17 | 2[a] |
| CIRNI | 25 | 0.28 | 0.22 | 0.08 | 0 | 0 | 0 | 1[a] |
| CIRNL | 58 | 0 | 0 | 0.10 | 0 | 0 | 0 | 1[a] |

[a] Number of major haplotypes.

stream of *dhfr*, except at the monomorphic +1.48-kb locus. In contrast, those isolates carrying the triple or quadruple mutations at *dhfr* had very low *h* values (0 to 0.28) at all microsatellite loci, indicating limited diversity in those isolates. Isolates carrying double mutations at *dhfr* had intermediate values of *h* (0.48 to 0.54) lying somewhere between those of the wild-type/single mutants and triple/quadruple mutants.

**Microsatellite haplotypes.** Different microsatellite haplotypes were found in isolates carrying wild-type *dhfr* and in those carrying single mutations; 8 haplotypes were found in 10 wild-type *dhfr* isolates, and 3 haplotypes were found in 5 single mutants (Table 1). In contrast, only two distinct microsatellite haplotypes (SEA/SEA variation and Melanesia/Melanesia variation) were observed in a total of 184 *dhfr* double-mutant isolates (Fig. 3). Identical or very similar haplotypes (SEA/SEA variation) were found in all *dhfr* triple or quadruple mutation-carrying isolates ($n = 83$), suggesting that *dhfr* triple and quadruple mutants evolved directly from the *dhfr* double mutant.

In Southeast Asia, only SEA/SEA variation haplotypes were observed. These haplotypes were also predominant in Melanesian countries, except Papua New Guinea, where 78% of isolates were of the Melanesia/Melanesia variation haplotypes. In Vanuatu, all isolates showed SEA/SEA variation haplotypes. One isolate carrying a hybrid of the SEA and Melanesia haplotypes (it was of SEA haplotype upstream and Melanesia haplotype downstream of *dhfr*, bp 200-194-176-100-205-111) was observed in the Solomon Islands.

**Genetic hitchhiking in *dhfr* double-mutant parasites from Papua New Guinea.** These results suggest that the *dhfr* double mutants present today in Melanesia emerged independently in Southeast Asia and Melanesia. To determine the history of these two lineages, we measured the extent of genetic hitchhiking, which is determined by the distance of reduced microsatellite variation around *dhfr*. For this purpose, the variance

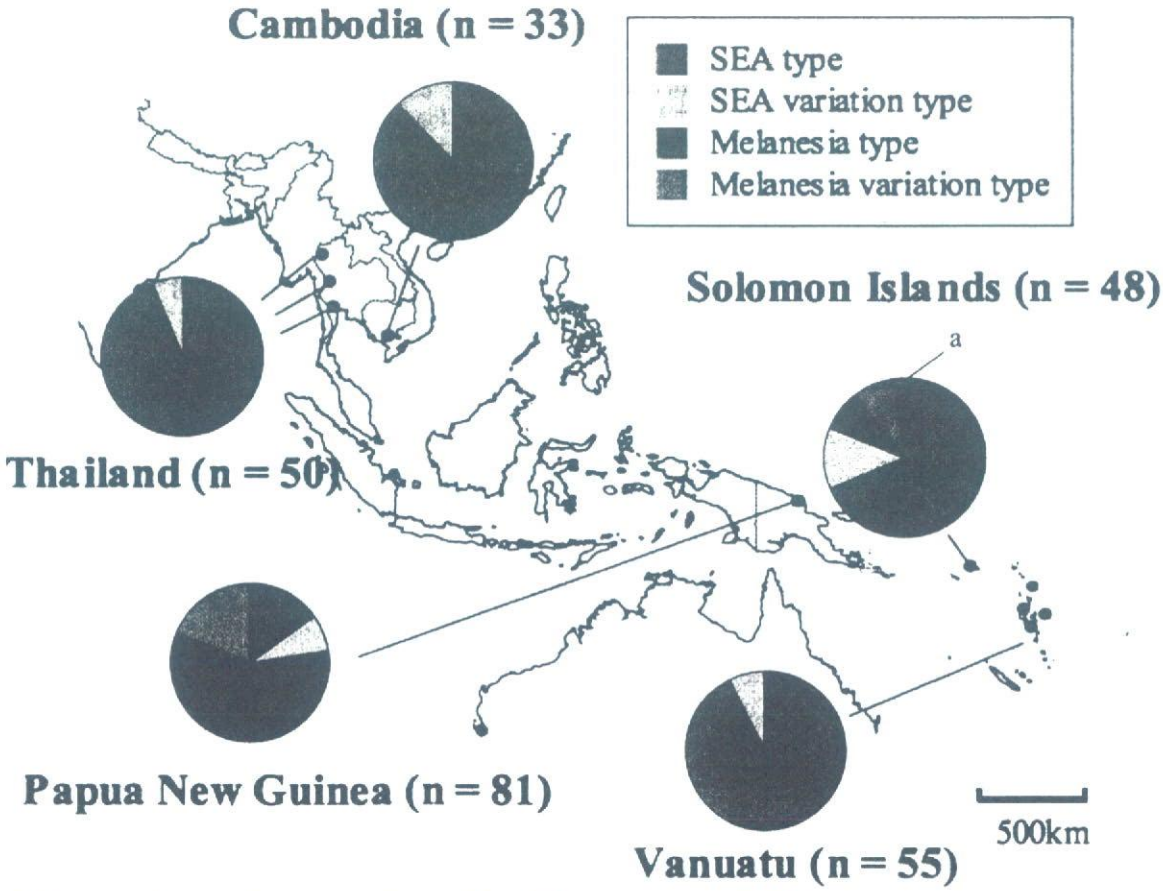


FIG. 3. Frequency of microsatellite haplotype flanking *dhfr* in *P. falciparum* isolates with *dhfr* double, triple, or quadruple mutants from Papua New Guinea, the Solomon Islands, Vanuatu, Cambodia, and Thailand. [a], isolate ($n = 1$) carrying a hybrid of the SEA and Melanesia haplotypes (it was of SEA haplotype upstream and Melanesia haplotype downstream of *dhfr*, bp 200-194-176-100-205-111).

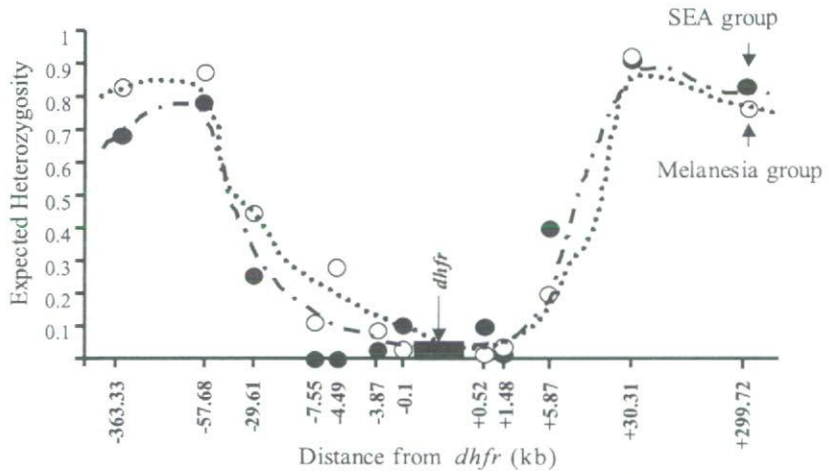


FIG. 4. Reduced microsatellite polymorphism near *dhfr* in *P. falciparum* isolates with CNRNI genotype from Papua New Guinea.

of *h* at 12 microsatellite markers spanning 363.33 kb upstream to 299.72 kb downstream of *dhfr* was measured for Papua New Guinean isolates with a *dhfr* double mutant displaying a SEA/SEA variation haplotype ($n = 17$) and those displaying the Melanesia/Melanesia variation haplotype ($n = 64$) (Fig. 4). The patterns of genetic hitchhiking in both haplotypes were similar within a distance of 58 kb upstream and 30 kb downstream of *dhfr*. These results suggest that these two lineages, both carrying the same point mutations (CNRNI), appeared coincidentally in Papua New Guinea.

## DISCUSSION

This study clearly shows that pyrimethamine-resistant *P. falciparum* evolved independently in Melanesia. It has previously been shown that a single lineage of pyrimethamine-resistant parasites arose in Southeast Asia, and subsequently spread to Africa (21). Pyrimethamine-resistant parasites from South America, which show *dhfr* genotypes different from those of other geographic areas, independently evolved in two foci within South America (2). Thus, there are at least four distinct independent origins of *dhfr* resistance presently identified. This is similar to the situation with chloroquine resistance, which has also been reported to have arisen independently a total of four times, once in Southeast Asia, twice in South America, and once in Melanesia (31).

A recent study has reported multiple origins of *dhfr* resistance within Kenya (6). However, care must be taken when basing conclusions about the origins of drug resistance on microsatellite variation from areas of high endemicity, such as Kenya. Two factors are likely to affect microsatellite polymorphism in areas of intense transmission. First, new microsatellite haplotypes are easily generated by meiotic recombination because of a high recombination rate and high prevalence of mixed-haplotype infections. Second, interallelic recombination within a microsatellite may generate new microsatellite alleles by an unequal crossing-over mechanism. Indeed, in a study by Roper et al. (21), nonuniform microsatellite haplotypes were noticeable around *dhfr* in pyrimethamine-resistant African isolates. These factors may be less important in areas of low transmission, such as Southeast Asia, and so do not compromise the conclusions of the present study.

Genetic hitchhiking reduces the expected heterozygosity of microsatellite markers around a selected gene, resulting in a valley of reduced variation. However, this association is easily broken down by recombination, resulting in a narrowing of the selection valley as the number of generations increases. In this study, we compared the selection valleys around the *dhfr* gene in two *dhfr* double mutants carrying SEA and Melanesia microsatellite haplotypes from Papua New Guinea. In both haplotypes, the microsatellite patterns within the valley were very similar from a distance of 58 kb upstream to 30 kb downstream of *dhfr*. The size of a selection valley is determined by several different parameters: the strength of the selection pressure on the mutant allele, the frequency of recombination, the transmission intensity, and the number of parasite generations since the emergence of the selected allele (16). In this analysis, these four parameters could be considered equal because all isolates were sampled from the same area. Thus, these results indicate that two ancestors of the *dhfr* double mutant in Papua New Guinea emerged coincidentally: one came from Southeast Asia, and the other arose independently within Melanesia. Although the appearance of the two resistant lineages emerged nearly simultaneously, we consider that the Melanesian-resistant type might have appeared slightly earlier than the influx of the SEA-resistant type. This is because if the SEA-resistant type migrated to Papua New Guinea earlier, it would have swept away microsatellite polymorphisms linked to the wild *dhfr*. Therefore, the possibility that a novel *dhfr*-resistant type having distinct microsatellite haplotypes appeared soon after the sweep in Papua New Guinea seems very unlikely.

The way drugs are used within regions of endemicity affects the generation and selection of resistant alleles. SP was widely used in Thailand and Cambodia during the 1970s and 1980s as the first-line treatment for uncomplicated malaria. In Melanesian countries, SP was introduced as a first-line treatment during the mid-1990s. Up until this time, pyrimethamine monotherapy was infrequently used for the treatment of malaria and other infections. It is difficult, therefore, to explain the nearly simultaneous emergence of pyrimethamine-resistant parasites

in this area. It is, however, possible to attribute the emergence and spread of the triple and quadruple *dhfr* mutants to the widespread use of SP in Southeast Asia during the 1970s and 1980s.

We consider that weak and persistent pyrimethamine pressure by medicated salt projects, a form of mass drug administration, may explain the first selection of pyrimethamine-resistant parasites (15, 25). In the late 1950s and early 1960s, medicated salt projects were carried out in four different endemic regions: Indonesian Papua, the Thailand-Cambodia border, Ghana, and Iran (15, 25). In Southeast Asia and Melanesia, this project was carried out from 1959 to 1962 and from 1960 to 1961, respectively. Pyrimethamine resistance in *P. falciparum* was reported within 3 months of the start of the project (1960) in Indonesian Papua (7). At the Thailand-Cambodia border, resistance also developed quickly and pyrimethamine was replaced by chloroquine beginning in 1961. A large number of people treated with subcurative doses of antimalarial drugs present ideal conditions for the emergence of drug resistance (4, 27, 28). This, combined with the long half-life of pyrimethamine (116 h) (29), would have facilitated the emergence of drug resistance within the areas covered by the medicated salt project: Indonesian Papua and the Thailand-Cambodia border.

The *dhfr* double mutant (CNRNI), which confers moderate resistance to pyrimethamine, is widely distributed in Africa, West Asia, and Melanesia. However, whether this mutant is regularly selected de novo or whether it has spread from a limited number of foci of emergence is not known. In the present study, only two distinct microsatellite haplotypes (SEA and Melanesia haplotypes) were observed in a total of 184 *P. falciparum* isolates harboring the *dhfr* double mutant from Papua New Guinea, Vanuatu, and the Solomon Islands, suggesting that the generation of two mutations at positions 59 and 108 in *dhfr* is not frequent. In laboratory isolates, key point mutations in *dhfr* have occurred at frequencies as high as $2.5 \times 10^{-9}$ per parasite replication, which predicts the generation of one mutant parasite in every malaria patient, assuming the number of parasites to be $10^{10}$ to $10^{12}$ in every infection (14). Consistently, the expected heterozygosities at microsatellite markers around *dhfr* were comparable between the wild-type and single *dhfr* mutant parasites. Thus, the initial mutation at position 108 in *dhfr* may occur relatively frequently (12, 20), but the generation and selection of an additional mutation at position 59 appear to be considerably less frequent. Mutations that render pathogens resistant to drug treatment are often associated with a loss of fitness (8, 11, 26). Resistant mutants may themselves develop compensatory mutations, which could then allow them to grow and survive in competition with wild-type forms (5, 26). The discrepancy between the frequent generation of the mutation at position 108 in *dhfr* and the rare occurrence of the *dhfr* double mutant as observed in this study may thus be reconciled by the requirement of complex compensatory mutations in a locus other than *dhfr* for restoring parasite fitness in natural populations.

In the present study, our samples of Papua New Guinea and Thailand were from individuals with clinical malaria, while samples from other sites were from cross-sectional studies of asymptomatic individuals. Symptomatic patients usually have higher parasite densities than do asymptomatic individuals. Thus, we cannot exclude the possibility that prevalences of microsatellite haplotypes may differ between isolates that cause disease and those that do not produce symptoms. However, we do not consider it very likely because there was no significant difference in the frequency distribution of genotypes of an antigen gene (*msp1*) between clinical patients and asymptomatic individuals in Melanesia (23).

In conclusion, this study provides strong evidence for the unique and independent origin of pyrimethamine resistance in Melanesia. The *dhfr* mutant, perhaps emerging from West Papua, has the same double mutations found in other geographic areas but distinct microsatellite haplotypes flanking the gene. Our results also show that the generation of double mutants with mutations at positions 59 and 108 of *dhfr* is a rare event, and this double mutation may be a first rate-determining step for the stable persistence of pyrimethamine resistance in *P. falciparum*.

## ACKNOWLEDGMENTS

We thank all the people who participated in the epidemiological studies for their kind cooperation; Hikota Osawa, Amane Suzuki, Miki Sakurai, Ilomo Hwaihwanje, and Francis Hombhanje for assistance in the field; and Culleton Richard for critical reading of the manuscript.

This work was supported by a grant-in-aid of the Ministry of Health, Labor and Welfare (H17-Sinkou-ippan-019); grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (17590380, 18390131, 18073013, and 18GS031); the Japan International Cooperation Agency (JICA) under the Integrated Cooperative Research for Malaria Control (ICRMC) project between the University of Papua New Guinea and Tokyo Women's Medical University; the Core University System Exchange Programme under the Japan Society for the Promotion of Science, coordinated by the University of Tokyo and Mahidol University; an institutional grant from the Swedish Foundation for International Cooperation in Research and Higher Education (STINT); and a project grant of the Swedish Research Council (VR, 2005-6836).

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