TESTING FOR ANTI-CIRCUMSPOROZOITE AND ANTI–BLOOD-STAGE ANTIBODIES
FOR EPIDEMIOLOGIC ASSESSMENT OF PLASMODIUM FALCIPARUM
INFECTION IN TRAVELERS

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Abstract. The purpose of this investigation was to assess the role of serology for establishing incidences of Plasmodium falciparum malaria and of exposure to P. falciparum in epidemiologic studies of travelers using chemoprophylaxis. The design was a prospective cohort study involving 548 short-term Dutch travelers to areas endemic for P. falciparum malaria. Sera were collected before departure and, together with the medical history, 2–6 weeks after return. All sera were tested for anti-circumsporozoite (CS) antibodies by an R32tet32–ELISA; sera of subjects reporting febrile illness during travel or after return or with anti-CS responses were tested for anti–blood-stage antibodies by an indirect fluorescence antibody test (IFAT). Five subjects (0.9%) reported P. falciparum malaria confirmed by thick blood smear examination (documented cases) and six (1.0%) reported treatment for malaria without a documented diagnosis (presumptive cases). Conversions in the IFAT were detected in six subjects, including all five documented cases and one presumptive case. Anti-CS antibodies were detected in seven subjects (1.3%), including three documented cases and four of 442 subjects with no history of fever or malaria treatment (0.9%). Incidence rates per 1,000 person-months of travel (95% confidence interval) of infection with P. falciparum, whether or not suppressed by chemoprophylaxis, were 16.9 (8–31) for all destinations and 91.6 (33–200) for West Africa. In epidemiologic studies of P. falciparum malaria in travelers, testing for antibodies to blood stages can increase the sensitivity and specificity of case detection; testing for antibodies to sporozoites may be useful for the assessment of exposure to P. falciparum in travelers using chemoprophylaxis, but the sensitivity is limited.

In most epidemiologic studies that assess risks of Plasmodium falciparum malaria or efficacies of prophylactic measures in nonimmune travelers, incidences are based retrospectively on cases of malaria that are confirmed by blood slide evaluation.2–5 This method is likely to underestimate true incidences of malaria because antimalarial treatment is frequently started without reliable parasitologic confirmation. In addition, this method will result in underestimation of the exposure to P. falciparum when the travelers studied take chemoprophylaxis. Since chemoprophylaxis suppresses the development of blood-stage parasites, infection by sporozoites will generally not progress to patent infection and clinical disease. Therefore, data based on blood slide results tend to reflect risks of breakthrough malaria or of malaria in individuals taking inadequate prophylaxis, and are confounded by drug resistance patterns and compliance with prophylactic regimens.

Serologic methods may have value in studies aimed at establishing malaria risks or efficacy of prophylactic measures in travelers by identifying individuals with recent exposure to sporozoites and/or by retrospective confirmation of diagnoses of malaria. Tests for antibodies to blood stages of P. falciparum are frequently used in seroepidemiologic studies of endemic populations.5–7 One report has illustrated the application of the indirect fluorescence antibody test (IFAT) for retrospective diagnosis of malaria in travelers who took stand-by antimalarial treatment.8 Antibody responses to sporozoites have been studied extensively in immune populations9–11 and in nonimmune individuals,12, 13 mostly by ELISAs based on synthetic or recombinant peptides from the repetitive domain of the circumsporozoite (CS) protein. Recently, a retrospective study reported a high prevalence (21%) of anti-CS antibodies in asymptomatic travelers returning from sub-Saharan Africa.14

We studied if testing for antibodies against the CS protein by R32tet32–ELISA and against blood stages by IFAT would improve the assessment of exposure to P. falciparum and the detection of cases of P. falciparum malaria in a cohort of travelers to endemic areas who were taking chemoprophylaxis.

SUBJECTS AND METHODS

The study was conducted at the travel immunization clinic of the Amsterdam Municipal Health Service. Between February 1991 and February 1992, all Dutch-speaking clients 15 years of age and older who intended to travel to tropical or subtropical destinations for 1–13 weeks were asked to participate. The analyses reported here included only subjects who traveled to areas endemic for P. falciparum malaria. The study was approved by the Service’s Board of Medical Ethics. Before departure, a blood sample was taken and a questionnaire that addressed travel history and plans was completed. All subjects were prescribed proguanil, 100 mg/day, and chloroquine, 300 mg/week, for chemoprophylaxis. The daily proguanil dose was doubled for all subjects enrolled after July 1991 due to a change in the chemoprophylaxis guidelines in The Netherlands. Subjects visited the clinic again within six weeks after return from the endemic area for a second blood sample and questionnaire. The post-travel questionnaire addressed travel itinerary, febrile illnesses, medical consultation, diagnosis, treatment, and use of chemoprophylaxis. Eligible travelers were included in the study only after having given written informed consent based on a information sheet on the study covering objectives, procedure, laboratory investigations, and confidentiality.

Data ascertainment and definitions. Travel destination

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was recorded as countries and grouped into five regions, largely corresponding to the classification used by the World Health Organization, with an additional subdivision of sub-Saharan Africa in a West and an East African region. Subjects who visited more than one geographic region were excluded since this would impair analysis by destination. Travel duration was recorded as the total travel time in weeks in areas meeting the inclusion criteria. Febrile illness was recorded as episodes of (subjective) fever of any duration occurring between the eighth day in an endemic area and the post-travel interview. Chemoprophylaxis compliance was recorded retrospectively as self-reported use.

Documented malaria was defined as febrile illness for which *P. falciparum* malaria had been diagnosed by blood slide examination. Diagnoses made in The Netherlands were confirmed by contacting the laboratory and/or the treating physician. Only written proof of the diagnosis was accepted. Presumptive malaria was defined as any episode of febrile illness for which antimalaria treatment had been taken, but no documented positive blood slide was available. Use of chemoprophylaxis was defined as complete when both proguanil and chloroquine had been taken from the day of departure to the endemic area until the post-travel visit or four weeks after return.

**Laboratory methods.** All serum samples were stored at −20°C. Pre- and post-travel samples of all subjects were tested for anti-CS antibodies in an ELISA using the synthetic antigen R32tet13 as described elsewhere. The antigen stock of 0.25 mg/ml was diluted 1:1,250 in phosphate-buffered saline (PBS) and 50 µl was applied per well of a polystyrene plate. Sera were diluted in PBS-Tween 20-0.01% polyethylene glycol (50 µl/well). Peroxidase-conjugated goat anti-human immunoglobulin was used at a dilution of 1:5,000 in PBS-Tween 20. Tetramethylbenzidine dichloroiodate tablets were diluted in phosphate-citrate buffer, pH 5.0 (60 µl/well); the reaction was stopped after 20 min by adding 4 N H2SO4 and read at 450 nm in a multi-scan plate reader. Negative and positive controls were included on each plate. The percent optical density (OD) was calculated based on the ODs of the control sera: (test serum)/(positive serum) × 100. A %OD < 10% was found to be the negative cut-off value.

Post-travel sera of all subjects who reported febrile illness as defined above or had a post-travel serum positive for anti-CS antibodies in the R32tet13-ELISA were tested twice by the IFAT for antibodies to asexual blood stages of plasmodia, once using late trophozoites and schizonts of cultured *P. falciparum* and, once using schizonts of *P. falciparum*. In addition, pretravel sera of subjects with IFAT-positive trophozoites and schizonts were applied to antigen slides, air-dried, and stored at −70°C. Serum dilutions, including negative and positive controls, were applied (1:20 and higher) for 30 min, followed by washing, drying, and incubation with sheep anti-human fluorescein isothiocyanate–conjugated immunoglobulin. The conjugate was diluted in PBS (pH 7.2) containing 0.05% Evans Blue. The slides were then washed with PBS, mounted with buffered glycerine (pH 9), and examined with a Leitz (Wetzlar, Germany) fluorescence microscope with a 50× objective. A negative cut-off value of 1:40 was used with either antigen.

Both the ELISA and IFAT were performed with a total immunoglobulin conjugate (including IgM). Separate studies with sera from donors who never visited malaria-endemic areas never showed positive results in either assay.

**Statistical methods.** Two-tailed Fisher’s exact tests were used for significance testing of 2 × 2 tables. Incidence rates (IRs) were calculated by dividing the number of subjects at risk by the total number of weeks traveled, and expressed per 1,000 person-months by multiplying the resulting figure by 4. The IRs of IFAT conversion were based on a person-time denominator that included all study subjects. Exact binomial and Poisson 95% confidence intervals (CIs) were calculated for proportions and incidence rates, respectively.

### RESULTS

Initially, 629 subjects were included who intended to travel to areas endemic for *P. falciparum* malaria. No post-travel data could be collected from 21 (3.3%) subjects. Excluded were 35 subjects (5.6%) who had spent less than one week in endemic areas or visited more than one geographic region; four subjects (0.6%) who had their post-travel visit within three weeks after departure; and 21 subjects (3.3%) who had their post-travel visit more than six weeks after return. The remaining 548 subjects (87.1%) were analyzed.

Characteristics of the study population are given in Table 1. Twenty-nine percent were less than 30 years of age; 12% were 50 years of age or older. Travel duration was six weeks or less for 88%. Southeast Asia was the region visited most; four countries (Indonesia, India, Kenya, and Thailand) accounted for 54% of the primary destinations. The proportion of subjects traveling during rainy seasons ranged from 22%
Characteristics and postravel serology results in subjects with documented and presumptive falciparum malaria, and of symptomless subjects who seroconverted for anti-sporozoite antibody responses, among 548 Dutch travelers to areas endemic for *Plasmodium falciparum*

<table>
<thead>
<tr>
<th>Subject</th>
<th>Destination</th>
<th>Onset of fever</th>
<th>ELISA</th>
<th>IFAT*</th>
<th>Compliance with chemoprophylaxis†</th>
<th>Interval‡ (weeks)</th>
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<tr>
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<td>Discontinued</td>
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</tr>
<tr>
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<td>+</td>
<td>Complete</td>
<td>8</td>
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<tr>
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<td>7**</td>
<td>West Africa</td>
<td>During travel</td>
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<td>10**</td>
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<td>11**</td>
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<td>+‡‡</td>
<td>Discontinued</td>
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</table>

* IFAT = indirect fluorescence antibody test.
† Self-reported compliance with malaria chemoprophylaxis regimen (weekly chloroquine plus daily proguanil).
‡ Number of weeks between arrival in malaria endemic area and post-travel serum sampling.
§ All cases of falciparum malaria diagnosed by blood slide examination.
¶ All cases of febrile illness treated with antimalarials without blood slide confirmation.
** Self-treatment of presumptive malaria.
# Treated by physician or other health care worker.
†† All subjects who reported no febrile illness (442 of 548) but had post-travel anti-circumsporozoite antibodies.
‡‡ Pretravel IFAT also positive.

and 29% for West and East Africa, respectively, to 53% for South America.

**Documented and presumptive malaria.** At the post-travel interview, febrile illness was reported by 106 (19.3%), documented malaria by five (0.9%) and presumptive malaria by six (1.1%) of 548 subjects. Of the documented cases, four had been diagnosed after return to The Netherlands. Blood slide examinations had been done in two of the presumptive cases. These were reported as probably negative, but treatment with chloroquine had nevertheless been given. Of the remaining four subjects with presumptive malaria, one was treated during travel by a physician, and three had taken self-treatment.

**Sporozoite antibodies.** No pretravel and seven post-travel sera (1.3%) tested positive for anti-CS antibodies by the R32tet,α-ELISA (Table 2). Anti-CS conversion was observed in three (2.8%) of 106 subjects who reported febrile illness and in four (0.9%) of 442 who did not. The three subjects who seroconverted with a history of febrile illness were documented malaria cases; all had positive IFAT results (see below). Of the four subjects who seroconverted without a history of febrile illness, one had positive pre- and post-travel IFAT results (no. 15 in Table 2). This subject had discontinued chemoprophylaxis during travel. The remaining three (no. 12–14) were IFAT negative and reported complete compliance with the chemoprophylaxis regimen. Of all seven ELISA-positive subjects, five (71%) had a history of previous travel to tropical areas, compared with 417 (77%) of 541 ELISA-negative subjects ($P = 0.66$).

**Blood-stage antibodies.** Of 106 subjects reporting febrile illness, six (5.7%) tested positive by IFAT for antibodies to blood stages of *P. falciparum* after return (Table 2), representing 1.1% of all 548 subjects. All six had negative pre-travel sera. Five were documented malaria cases. The remaining subject was a presumptive case; he had taken halofantrine during an episode of fever without blood slide examination. Of four additional subjects with positive post-travel ELISA results, one also tested positive in the IFAT, both in the post- and pre-travel serum (subject no. 15). He had a history of frequent travel in East Africa.

Results of IFAT testing using *P. falciparum* antigen were positive for all five documented malaria cases, but titers were lower than in the IFAT using *P. falciparum* antigen. The IFAT results using *P. falciparum* antigen were negative for the six subjects with presumptive malaria, including the one with a positive IFAT result using *P. falciparum*, and for the four who tested positive in the ELISA without a history of febrile illness.

**Time intervals.** The median interval between onset of fever and post-travel serum sampling was four weeks (range = 2–9) for all presumptive and documented cases, and two weeks (range = 2–7) for cases with IFAT conversion. The interval between the first possible exposure to sporozoites (i.e., arrival in the endemic area) and the post-travel sampling was eight weeks or less for seven of seven ELISA-positive subjects (100%; Table 2), compared with 133 (25%) of 541 ELISA-negative subjects ($P = 0.20$).

**Compliance with chemoprophylaxis.** Twenty-three subjects (4%) had taken no prophylaxis and 21 (4%) had taken either no chloroquine or no proguanil. Prophylaxis was discontinued during travel by 79 (14%) and within four weeks after return by 98 subjects (18%). Adequate chemoprophylaxis was reported by 326 subjects (60%); among subjects traveling to West Africa, this was 32 (54%) of 60.
**Incidences and geographic distribution.** Table 3 shows incidence rates per 1,000 person-months of travel (pmt) of infection with *P. falciparum* as established by positive blood slide examination, seroconversion to anti-CS antibodies, seroconversion to anti–blood-stage antibodies, and seroconversion in either serologic test (combined serology). Positive combined serology was found in 10 of 548 subjects (1.8%, 95% CI = 0.9–3.3%), of whom six had traveled to West Africa (6 of 60 or 10.0%, 95% CI = 3.8–20.5%). The overall IR of *P. falciparum* infection was 8.5/1,000 pmt when based on blood slides only, but 16.9/1,000 pmt when based on combined serology. For West Africa, this was 45.8/1,000 pmt and 91.6/1,000 pmt, respectively. Of subjects who converted in either test (combined serology), four (40%) of 10 had traveled during the rainy season, compared with 195 (36%) of 538 with negative serology only (P = 1.000).

Of 415 subjects for whom the time interval between arrival in the endemic area and the post-travel serum sampling was eight weeks or less, nine (2.2%, 95% CI = 1.0–4.1) had positive combined serology for an overall IR (95% CI) of 26.0/1,000 pmt (11.9–49.3/1,000 pmt). Among subjects traveling to West Africa, these figures were five of 46 (10.9%; 3.6–23.6%) and 144.9/1,000 pmt (47.1–338.2/1,000 pmt), respectively.

**Comparative sensitivity.** Combined serology identified 10 *P. falciparum* infections. Assuming that all serologic conversions reflected true infections, the sensitivity (95% CI) for retrospective detection of infection with *P. falciparum*, regardless of whether clinical malaria ensued and compared with the combined serology results, was five of 10 (50%, 19–81%) for blood slide examination, six of 10 (60%, 26–88%) for testing for anti–blood-stage antibodies, and seven of 10 (70%, 35–93%) for testing for anti-CS antibodies.

**DISCUSSION**

Our study demonstrates that serologic methods can be used to improve the sensitivity for assessing exposure to *P. falciparum* in travelers. Detection of antibody responses to blood stages and of responses to sporozoites showed that incidences of infection with *P. falciparum* (both suppressed and patent) were higher than those estimated on the basis of blood slide examinations alone. The tests differ in their interpretations, their limitations, and the types of studies for which they might be useful.

Anti–blood-stage antibody responses indicate recent blood-stage infection, which is generally considered to result in clinical malaria in nonimmune travelers. Apart from the five cases identified by blood slide examination, seroconversion was demonstrated in one presumptive case. This is likely to reflect a true *P. falciparum* infection since the serum tested negative with schizonts of *P. fieldi* that show stronger cross-reactivity with the remaining human plasmodia.17 In one subject, the pretravel IFAT result was positive, and 77% of the subjects had traveled to tropical destinations previously. The study population may therefore not have been entirely nonimmune to *P. falciparum*. However, even in travelers with some immunity, *P. falciparum* malaria without any fever will be unlikely, and we do therefore not expect to have missed malaria cases by only testing the subjects who reported febrile illness.

One may argue whether the identification of one additional case merits the use of this test. However, the IFAT also identified 100 of 101 cases of undiagnosed febrile illness, including five of six in which malaria treatment had been given, as most likely caused by other pathogens. Including all six presumptive cases as *P. falciparum* malaria would have severely overestimated the incidence. This test may therefore be useful for retrospective confirmation of malaria in travelers, particularly in studies in which a relatively large number of malaria cases is expected to remain undiagnosed by blood slide examination (e.g., in long-term travelers).

The IFAT results may have included false-negative ones, especially among subjects who received malaria treatment and had long intervals between onset of symptoms and serum sampling. However, all subjects with positive blood slides had detectable anti–blood-stage antibodies, most of them at two weeks after onset of symptoms, when maximum titers can be expected.17, 18 In other studies, the IFAT has shown high sensitivity for retrospective identification of *P. falciparum* malaria in nonimmune individuals, especially when homologous plasmodia are used as antigen, and in most cases of *P. falciparum* malaria in nonimmune individuals titers remain detectable for 1–6 months.8, 18 Infection with non-*P. falciparum* plasmodia is unlikely in these subjects since they tested negative with *P. fieldi* antigen.

Of our total study population, 1.3% showed antibody responses to the CS protein of *P. falciparum*, indicating recent infection by sporozoites. Four of seven positive subjects had no history of febrile illness or malaria treatment. Three of
these reported adequate chemoprophylaxis. The fourth subject may have been protected from clinical malaria by chemoprophylaxis before discontinuation; some degree of immunity cannot be ruled out because he had previously traveled to East Africa and anti-blood-stage antibodies were detected in his pretravel sample. Assuming that most sporozoite inoculations into nonimmune individuals will progress to clinical malaria, this suggests that this technique can identify infections that were successfully suppressed by chemoprophylaxis and that it may be used to assess true exposure to infectious parasites in travelers taking chemoprophylaxis.

False-positive results are unlikely because the R32tet-ELISA is highly specific for exposure to Plasmodium falciparum sporozoites. Although the sensitivity of this test for detecting anti-CS antibodies is high compared with other methods, the sensitivity of detecting such antibodies for the assessment of recent exposure seems to be limited. Antibody responses to sporozoites are more variable than those to blood-stage parasites; this is probably due to the small numbers of sporozoites that are injected and their short lift-span in the circulation. Our finding that only three of six subjects with a blood-stage antibody response had detectable anti-CS antibodies is consistent with the detection of anti-CS responses in 42–57% of nonimmune P. falciparum malaria patients in other studies. It has been suggested that the development of a detectable anti-CS response is dose-related and requires multiple inoculations, whereas a single sporozoite inoculation could be sufficient to induce clinical malaria. This would imply that the positive ELISA results in our study represent multiple exposures to P. falciparum, whereas the negative subjects who had positive blood slides and/or IFAT results had only one or few inoculations. However, sporozoites have been shown to be highly immunogenic, and anti-CS titers decrease rapidly after the onset of symptoms. Therefore, antibody responses may have been initially present in all infected subjects, but remained undetected as they had decreased below the detection threshold by the time they were measured. This hypothesis is supported by our finding that positive ELISA results were only found in samples collected shortly after the malaria attack, and by the increase in incidence of seroconversion when the analysis was restricted to subjects whose samples were taken within eight weeks after the first possible exposure. The percentage of subjects with anti-CS antibodies was much lower than the 21% reported in a retrospective study of German travelers returning from sub-Saharan Africa that used an ELISA based on the synthetic CS protein [NANP]18. Apart from possible differences in the sensitivity and specificity of the tests, this may be due to differences in the selection of the study population, to the shorter travel time and thereby shorter exposure in our study (mean duration = 4.3 versus 7.5 weeks), and to pre-existing positive reactions in some of the German travelers due to previous exposures. Since our study was prospective, and anti-CS antibody test results before the journey were negative, the results are more likely to reflect true incidences of P. falciparum sporozoite inoculation in nonimmune travelers. Nevertheless, even though the frequency of previous travel to tropical areas did not differ between ELISA-positive and ELISA-negative sub-

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