Detection of very low level Plasmodium falciparum infections using the nested polymerase chain reaction and a reassessment of the epidemiology of unstable malaria in Sudan

ARTICLE in THE AMERICAN JOURNAL OF TROPICAL MEDICINE AND HYGIENE · MAY 1996
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DETECTION OF VERY LOW LEVEL \textit{Plasmodium falciparum}
INFECTIONS USING THE NESTED POLYMERASE CHAIN REACTION
AND A REASSESSMENT OF THE EPIDEMIOLOGY OF UNSTABLE
MALARIA IN SUDAN

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Abstract. We have used the nested polymerase chain reaction (PCR) to assay for low level \textit{Plasmodium falciparum} infections that were below the threshold of detection of blood film examination. This revealed a substantial group of asymptomatic, submicroscopically patent infections within the population of a Sudanese village present throughout the year although clinical malaria episodes were almost entirely confined to the transmission season. In our September, January, April, and June surveys, the PCR-detected prevalences were 13%, 19%, 24%, and 19%, respectively. These figures reveal a much higher prevalence of dry season infection than previous microscopic surveys have indicated. Furthermore, 20% of a cohort of 79 individuals were healthy throughout the September to November transmission season but were PCR-positive for \textit{P. falciparum} in a least one of a series of samples taken in the ensuing months. Levels of exposure to \textit{P. falciparum} infection were therefore higher than was previously believed in this region, highlighting the fact that many individuals were infected but healthy for most of the year. The reservoir parasite population was thus larger and more stable than previously thought, a finding that is consistent with the high levels of genetic variation at polymorphic loci reported from analysis of \textit{P. falciparum} parasites in this area.

The epidemiology of \textit{Plasmodium falciparum} malaria in central and eastern Sudan is considered to be at the unstable end of the spectrum described by Macdonald.\textsuperscript{1} The major features of unstable malaria are that transmission is absent during much of the year and may not occur at all in certain years. A resurgence of transmission may then cause epidemics during which the entire population is vulnerable to malaria. Immunity among adults is not pronounced and mortality is not confined to young children and pregnant women, as tends to be the case in areas of holoendemic malaria. Over the long term, it is therefore possible that malaria causes as much morbidity and mortality in areas of unstable transmission as it does in areas where entomologic inoculation rates are much higher.\textsuperscript{2,3}

In a preliminary survey of antimalaria immunity in Daraweesh village in eastern Sudan carried out in 1988, Theander and others\textsuperscript{4} found that no one had malaria symptoms or a positive blood film at the end of the dry season (June). Nor was there any significant difference in the prevalence rates of \textit{P. falciparum} infection between age groups during the transmission season (September–November). During the 1988 transmission season, approximately 30% of the Daraweesh residents had a symptomatic and confirmed case of \textit{P. falciparum} malaria.\textsuperscript{4,5} The instability of malaria in this region was emphasized when from October 1989 until June 1992, there was a severe drought as the seasonal rains failed for two years in succession. This disrupted transmission and there were no malaria cases in a closely observed study cohort (130 individuals) and less than 10 cases in the entire village during the 1990–1991 and 1991–1992 seasons.\textsuperscript{5}

The traditional interpretation of this type of malaria situation is that the instability of transmission and the low levels of parasite prevalence prevent the development of immunity in the population. As a result, infection is expected to lead to clinical disease. However, studies monitoring human immune responses to malaria antigens indicate that the Daraweesh situation is more complicated than this. The T cell responses to \textit{P. falciparum} antigens are reduced in both clinically ill and in healthy, apparently aparasitemic adults during the transmission season.\textsuperscript{3,6} Increases in specific antimalarial antibody titers over the course of the 1993 transmission season also occurred in a large proportion of the closely observed study cohort in Daraweesh, despite the fact that they had no clinical malaria episode or symptomatic complaints.\textsuperscript{6}

These observations indicate that more of the population are exposed to \textit{P. falciparum} infections than actually experience clinical malaria. This implies the population has a higher degree of immunity than expected and that many individuals control infections to such an extent that they are normally asymptomatic with low parasitemias that are difficult to detect. To test this hypothesis and measure the frequency of very low parasitemia infections in the Daraweesh study cohort, we have assayed blood samples taken at different points in the year using a sensitive nested polymerase chain reaction (PCR) technique for the detection of \textit{P. falciparum} DNA.

MATERIALS AND METHODS

Study area. The study was conducted in the village of Daraweesh in Gedaref State in eastern Sudan, approximately 80 km from the Ethiopian border. The region is mainly supported by large-scale sorghum cultivation, dependent on June–September rains. Malaria is loosely classified as me-
soendemic and transmission is marked seasonal, with cases peaking in October and November in frequente outbreaks.5 The village itself has approximately 350 inhabitants, the descendants of a Fulani group originating in what is now Burkino Faso who settled in Sudan a century ago. Plasmodium falciparum is the predominant species of malaria parasite and accounts for 95% of all malaria cases in the area. Although a few cases of P. malariae and P. vivax have been seen, their contribution to malaria morbidity is thought to be minor. Anopheles arabiensis is the main vector in this area of Sudan.7 Since 1988, the inhabitants of Daraweesh have agreed to participate in a study of the factors underlying clinical malaria and the development of antimalarial immunity in an area where transmission is restricted by the 9–10-month dry season.

Malaria case detection in the study population. This study was based upon the observation of a volunteer cohort whose clinical history has been followed since 1990. Cohort members were permanent village residents from families without the sickling allele of the a hemoglobin gene. Active detection of malaria infections within the cohort (and the village) took place from the beginning of September until mid-January each year. At the beginning and end of this period, cohort members donated a blood sample for immunologic analysis and screening for malaria parasites. Passive case detection occurred through villagers reporting to the study team if they had complaints or symptoms suggestive of malaria; a blood smear was then taken from these individuals and examined. Clinical malaria was defined as a parasite positive blood slide together with symptoms suggestive of malaria. Patients with positive blood films were treated with chloroquine. Sulfadoxine/pyrimethamine treatment was used for chloroquine-resistant infections.

Malaria case detection in the village as a whole occurred through a clinic run by a health team present in the village at least every second day. A village health worker resident in the village supplemented this coverage by being available for consultation and by regularly visiting all the family compounds in the village. In addition, each cohort member was visited by members of the health team every two weeks, oral temperatures were taken, and they were asked whether they had felt unwell during that period. No antimalarial prophylaxis was used in the village and market purchase of medication was unnecessary since the health team provided free medicine and treatment for Daraweesh and the surrounding villages.

Malaria parasite detection. Microscopy. Thick blood smears were taken from the whole cohort in September 1993, January 1994, April 1994, and June 1994. At each time point, thick and thin films were stained with Giemsa and examined microscopically. Films were considered negative after examination of 200 microscopic fields without detection of parasites.

Preparation of P. falciparum DNA for PCR analysis. Blood samples of two kinds were analyzed in the course of this study. Venous blood samples were taken from all members of the cohort in September 1993, January 1994, and June 1994. Lymphocytes were separated from a packed red blood cell pellet and 1 ml of packed red blood cells were retained for use in the DNA extraction and PCR analysis. During the April cross-sectional survey, smaller quantities of blood (0.1 ml) were taken by fingerprick sampling. The whole blood from these samples was used directly for the DNA extraction and PCR analysis. All samples were stored and shipped from the field in liquid nitrogen and stored at −70°C prior to analysis. From both types of samples, PCR-quality DNA from both types of sample was prepared using the rapid hemoglobin extraction protocol.8 A total of 20 µl of whole blood or packed red blood cells were vortexed in 0.5 ml of ice-cold 5 mM sodium phosphate, pH 8.0, which punctures erythrocytes and releases soluble components, notably hemoglobin. Insoluble material was then pelleted by centrifugation in a microfuge tube. Pellets were washed and resuspended in 0.5 ml of 5 mM sodium phosphate twice more. The pellet was then resuspended in 50 µl of sterile water and boiled for 10 min. After a final centrifugation, the DNA-containing supernatant was removed and stored at −20°C prior to PCR analysis.

Detection of parasites by the PCR. Plasmodium falciparum DNA was detected by nested PCR amplification of the small subunit ribosomal RNA gene using the primers and cycling parameters described by Snounou and others.3, 10 Outer PCR reactions were carried out with 4 µl of DNA (prepared as above) in 20-µl reactions containing 2 mM MgCl2, 50 mM KCl, 10 mM Tris HCl, pH 8.3, 0.1 mg/ml of gelatin, and 125 µM of each of the four deoxynucleotide triphosphates. Each primer was present at a concentration of 250 nM and 0.5 units of AmpliTaq polymerase (Perkin-Elmer Cetus, Norwalk, CT) per reaction were used. One microliter of the product of the outer reaction was used as a template for the second reaction. A negative control, to which no DNA had been added, and a positive control, to which P. falciparum clone 3D7A DNA had been added, were included in each set of amplification reactions. The PCRs were analyzed on 1.5% agarose gels and visualized after staining with ethidium bromide.

RESULTS

Sensitivity of nested PCR parasite detection. To establish the sensitivity of the nested PCR assay using our sample preparation and reaction conditions, fixed numbers of P. falciparum (clone 3D7A) parasites were used in a limit dilution detection assay. Both aparasitemic, cryopreserved Sudanese blood and fresh, uninfected blood were used as diluents. The DNA for PCR amplification was extracted and detection sensitivity in the cryopreserved samples was compared with that obtained when dilutions were performed with fresh blood.

Nested PCR detection of the P. falciparum ribosomal DNA gene fragment in fresh blood is shown in Figure 1A. In Figure 1B, detection of the same numbers of parasites is assayed after dilution in cryopreserved uninfected blood. Figure 1C shows a typical stained agarose gel of the products of the second amplification reaction in a nested PCR screen of a group of Daraweesh blood samples. Using 3D7A parasites added to 20 µl of fresh blood, specific amplification product was observed in all samples where the expected number of parasites was one or higher. Bands present in the wells containing 0.5 and 0.2 estimated parasite genomes per sample indicate that the initial culture parasitemia was slightly underestimated. In Figure 1B, using 20 µl of cryopreserved packed red blood cells (with approximately twice the
normal hematocrit) from a Daraweesh sample as diluent, the assay was somewhat less sensitive and a specific amplification product was observed in all reactions where the expected number of parasites was greater than 10.

The amount of human DNA (and parasite DNA if present) in each reaction was approximately equivalent to that in 4 μl of blood in the cryopreserved samples. *Plasmodium falciparum* detection in these field samples was therefore approximately 5–10 times less sensitive than the observed detection limit of single parasite genomes obtained with fresh material. Therefore, a conservative detection limit of 10 parasites in 4 μl (2.5 parasites/μl) of human blood is assumed in this study, which corresponds to a parasitemia of approximately 0.00005% (assuming 5 × 10^6 erythrocytes/μl of blood). This indicates that we are detecting parasitemias at the bottom end of a range of between one and 50 parasites/
Seasonal incidence of clinical and asymptomatic infections. Asymptomatic infections in this study were detected by both microscopy and by nested PCR amplification of the rDNA target sequence. The numbers of symptomatic and asymptomatic infections at four points during the 1993–1994 transmission season are shown in Table 1. The table shows the number of cases at each point with the results of September to January case surveillance added to emphasize the continuity of the survey. Cases are categorized as symptomatic, asymptomatic but microscopically positive, and asymptomatic, microscopically negative but PCR positive. The sum of these numbers is given in the last column of the table. More asymptomatic infections were detected by PCR than by blood film examination and this reflects the greater sensitivity of this method. It also indicates that parasitemias are contrasted with the total numbers of clinical cases of malaria. No blood film–positive cases were PCR negative; therefore, the sum of these values (given in the last column of the table) is the total number of the cohort at that time point who had a Plasmodium falciparum infection detected by either blood film or PCR.

µl of blood. Such samples constitute those low and very low parasitemias most likely to be missed in examination of Giemsa-stained thick blood films, which have a sensitivity threshold of approximately 10–20 parasites/µl, even in experienced hands in good laboratory facilities.11

### Table 1

<table>
<thead>
<tr>
<th>Asymptomatic infections</th>
<th>Asymptomatic, but positive blood film, but PCR-positive</th>
<th>Total infections (total sampled)</th>
</tr>
</thead>
<tbody>
<tr>
<td>September 1993</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>September–December 93</td>
<td>24</td>
<td>Screening of cohort not done during transmission season</td>
</tr>
<tr>
<td>January 1994</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>April 1994</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>June 1994</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* The same 79 people were present in each sample with the exception of June when six people were unavailable and the total sample number was reduced to 73. The cohort are not screened for asymptomatic infections during the September to January period. The total number of asymptomatic but blood film–positive and polymerase chain reaction (PCR)–positive infections are contrasted with the total numbers of clinical cases of malaria. No blood film–positive cases were PCR negative; therefore, the sum of these values (given in the last column of the table) is the total number of the cohort at that time point who had a Plasmodium falciparum infection detected by either blood film or PCR.

Continuity of infection in individuals. A history of malaria infection status in the cohort during the study period is shown in Figure 2. This details the clinical incidence of malaria among individuals and illustrates how this related to their infection status in the preceding September (A) or in subsequent time points; January (B), April (C), and June (D). The upper bar of Figure 2A divides the cohort into three infection classes, those people with symptomatic infection, those asymptomatically infected, and those without detectable infection. The expansion from the upper bar shows whether these individuals went on to develop clinical malaria during the subsequent months, as detected by morbidity surveillance in Daraweesh.

In Figures 2B–D, this process is reversed and the accumulated information on those who have and have not suffered a clinical malaria attack is summarized in the upper bar. The expansion from the upper bar then details the infection status of the individuals at the January, April, and June survey points. The following points can be summarized from these data.

1) Asymptomatic infection in September might be expected to influence the likelihood of an individual having a subsequent clinical episode if the infection was in the incubation phase of infection, or conversely if it conferred protection against illness. Three of the eight asymptotically infected individuals subsequently developed clinical symptoms of malaria. This proportion does not deviate from the expected as predicted from the overall incidence of clinical episodes when tested using a chi-square test. Of the three cases, none complained of illness until at least two weeks after the PCR prevalence samples were taken, and in one case symptoms appeared 32 days later.

2) Thirty-five percent of the people who experienced a clinical episode during the transmission season had asymptomatic infections in January. This compares with an 8% incidence of asymptomatic infection among those who had no clinical record of malaria. A chi-square test (excluding the two symptomatic January cases) showed that the incidence of asymptomatic January infections was significantly (P < 0.005) higher among people with a recent clinical experience of infection than would be predicted to occur by chance alone.

3) The April infection status results again show that those who had experienced a clinical episode in the transmission season were more likely to be carrying an asymptomatic infection in April, although all of these individuals had received chemotheraphy and all had been clinically cured. A chi-square test showed this tendency to be statistically significant (P < 0.001). The continuing association of clinical malaria with subsequent asymptomatic infection is mainly due to the persistence of asymptomatic infection from January through April in the same patients. A chi-square test showed that people with asymptomatic infection in January were significantly more likely to remain infected in April than would be expected by chance alone (P < 0.001).

4) By June, the association between clinical experience of malaria and subsequent PCR positivity disappeared and a chi-square test showed that the proportion of asymptomatic infections in the group with clinical history was not significantly different from that which would be expected by chance alone (P < 0.1). The continuity of asymptomatic infection from April to June was significant (P < 0.005). It is interesting to note that of the eight asymptomatic cases found among the group who had not had a malaria episode that year, six had no evidence of infection at any of the preceding survey points.

**Age distribution of symptomatic and asymptomatic infections within the study cohort.** To examine the influence of age upon experience of *P. falciparum* infection during this study, individuals were again divided into three cate-
EPIDEMIOLOGY OF UNSTABLE MALARIA IN SUDAN

Morbidity Prevalence

<table>
<thead>
<tr>
<th>symptom</th>
<th>infection</th>
<th>no symptoms</th>
</tr>
</thead>
</table>

PCR Prevalence

<table>
<thead>
<tr>
<th>PCR negative</th>
<th>asymptomatic</th>
<th>PCR positive</th>
</tr>
</thead>
</table>

FIGURE 2. Relationship between *Plasmodium falciparum* infection and clinical malaria episodes in individuals in Daraweesh from September 1993 to June 1994. The clinical incidence of malaria among individuals is related to their infection status in the preceding September (A) or in subsequent time points: January (B), April (C), and June (D) (see text). The total number of cohort members who had a clinical malaria attack over the entire period was 28, of whom two had malaria at the initial cross-sectional survey in September 1993 (2A) and two were ill during the January 1994 survey (2B). As the overall sample sizes in Table 1 show, six of the cohort were absent during June 1994, thus reducing the sample numbers in that survey (2D). PCR = polymerase chain reaction.

categories of infection status; those with clinical experience of infection, those without clinical experience but who had an asymptomatic infection, and those with neither clinical nor asymptomatic infections. Figure 3 illustrates the overall age structure of the study cohort with people within each age class subdivided according to infection status. There are limitations placed on any analysis of this data because the overall sample size is small and because of the uneven representation within the different age classes. Given these caveats, the data shown in Figure 3 do not indicate a preponderance of any category of infection status in any age class. It may therefore be tentatively concluded that the influence of age upon susceptibility to clinical infection or to asymptomatic tolerance of parasite infection was small.

DISCUSSION

The interaction between the epidemiology of malaria and the development of human immune responses to the disease in areas of low and unstable transmission is not well-understood. In our study village, sensitive nested PCR parasite detection assays have revealed an unexpected picture of year-round presence of *P. falciparum* among the population. Contact between parasites and humans was originally thought to be largely restricted to cases of clinical malaria occurring in short seasonal outbreaks. It now appears that the extent of contact between human and parasite populations is wider and more prolonged. The PCR assays indicate that asymptomatic, low-density *P. falciparum* infections occurred year-round and in our September, January, April, and June surveys, the prevalences were 13%, 19%, 24%, and 19%, respectively. These are much higher levels of infection than previously diagnosed microscopically. Similar large increases in the estimated prevalence of malaria infection obtained using PCR techniques have also led to a re-evaluation of the malaria epidemiology of a village in a holoendemic area of Guinea-Bissau.

These results compliment previous immunologic data from this study, which indicate that asymptomatic infection rates during the transmission season itself are surprisingly high. Antibody titers to blood-stage parasite antigen were at least four times higher at the end of the 1993–1994 transmission season than at the beginning in 90% of the individuals sampled. Only 33% of these individuals had reported clinical symptoms.

Genetic diversity among *P. falciparum* parasites isolated from clinical cases collected in Daraweesh and neighboring villages have also been shown to be extremely high. A reasonable explanation for this is that these isolates were drawn from a large parasite population. If parasite
prevalence was low prior to the transmission season, then it would be expected that outbreaks would be seeded from a limited number of sources and genetic diversity in parasites isolated from clinical infections would reflect this. The high level of genetic diversity observed in clinical isolates is more consistent with a large effective population size of the local parasite population than with the expansion of a small number of genetic lineages.

In the January and April 1994 surveys, PCR-positive samples occurred more frequently among those with experience of a clinical infection during the transmission season. It is possible that these asymptomatic infections were a chronic continuation of the initial infection despite treatment. This phenomenon has been observed in Thailand and was considered to be caused by persistence of drug-resistant parasites.\(^\text{10}\) The fact that the incidence of asymptomatic infection among this group had decreased to a nonsignificant level by June implies that few of these infections persist through the dry season. An alternative explanation for the association is that this group is more susceptible to reinfection due to factors such as proximity to breeding sites or preference for outdoor sleeping. This hypothesis presupposes that transmission continues during the dry season, a possibility that will be discussed later.

The observation that 20% of the cohort were healthy throughout the study but were parasite-positive in at least one of the January, April, or June samples indicates that many asymptomatic infections were not simply sequels to recent clinical episodes. It may suggest that this proportion of the population has some protective clinical immunity at least against certain strains of parasites. There is no trend for the age distribution of these cases to be higher than that of symptomatic cases, indicating that any such protection has no strong age-dependent component.

In the series of samples following the transmission season, many individuals were found to convert from PCR-positive to PCR-negative without any clinical malaria episode. Whether this reflects immune control and clearance of a new infection or simply fluctuations above and below a detection threshold of a chronic infection is not known.

We found that nine people who were not PCR-positive in January were subsequently positive in April, and seven people negative in April were then found to be positive in June. The fact that people became parasite positive during the exceptionally hot and arid Sudanese dry season is surprising but could be explained in one of two ways. As considered above, fluctuations in parasite density around the threshold limit of PCR detection may well be characteristic of chronic infections. Alternatively, these transitions from negative to positive could be the result of new infections arising through the inoculation of sporozoites by vectors feeding during the dry season. *Plasmodium falciparum* was detected in June in six individuals who had no history of clinical or asymptomatic infection at any of the earlier time points. Furthermore, it has been shown that during the dry season, the female *An. arabiensis* of central Sudan can undergo an incomplete reproductive diapause induced by changes in humidity and temperature during which time they continue to feed, predominantly on human blood, but take only partial blood meals.\(^\text{18,19}\) The continuation of feeding behavior in these long-lived forms means that the transmission of *P. falciparum* could potentially continue at a low level throughout the dry season. However, dry season female *An. arabiensis* are extremely difficult to find in the study area (Hamael AA, unpublished data), and the infectivity of very low parasitemia, submicroscopic infections to biting anophelines is unknown. The existence of dry season malaria transmission and the possible significance of its role in the local epidemiology of *P. falciparum* have yet to be established.

Asymptomatic malaria infection could be a significant health problem under two circumstances in this epidemiologic setting. First, there may be consequences for the individual of harboring such an infection. Asymptomatic infections may be in the process of being controlled and eliminated but they may also be capable of conversion to clinical disease. Second, there are the consequences to the general population of the presence of individuals harboring such infections. The obvious danger is that such asymptomatic carriers are a reservoir of infection capable of infecting others both during and after the nine-month dry season.

Asymptomatic malaria infections are of course common in holoendemic transmission zones in the wet tropics.\(^\text{20}\) It is the long, hot Sudanese dry season that imposes severe limitations on transmission that highlights the problem of the nature of the reservoir of infection and the significance of asymptomatic carriers. Although the restricted conditions of
malaria transmission in Sudan have allowed us to define the problem more precisely, the questions posed above are largely unanswered. Accurate determination of the duration and clinical course of individual asymptomatic infections will require continuous sampling over a longer period and genetic marking of the genotype(s) present throughout individual infections. Clonal antigenic variation in *P. falciparum*, which may maintain low level parasitemias by enabling them to escape immune clearance, also needs to be investigated in these epidemiologic studies.21-23

Acknowledgments: We thank Ababae Gebre for excellent field and laboratory assistance, Abdelhameed Dardiry Nugud and Amal Awad Hamael of the Department of Medical Entomology at the University of Khartoum for entomologic assistance and discussions, and David Walliker and Lisa Ranford-Cartwright for malarialogic discussions. We also thank Georges Snounou for generous assistance with the nested PCR primers and detection protocols.

Financial support: This work received support from a UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases grant (no. 910446) to David E. Arnot and Cally Roper. Field work in Sudan was also supported by the Danish International Development Agency (DANIDA grant 104. Dan. SL/401). Lars Hvid is a Weimann senior research fellow and David E. Arnot is a Senior Fellow of the Wellcome Trust, who also supported this work through a Biomedical Research Collaboration Grant to the Edinburgh and Copenhagen groups.

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