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Malaria in São Tomé and Príncipe: parasite prevalences and vector densities☆

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Abstract

A cross-sectional survey was carried out in 16 localities on the island of São Tomé and three on the island of Príncipe, at the end of the rainy season of 1997, to determine malaria prevalence and vector densities. Blood samples from 664 inhabitants of all ages were examined by optical microscopy (OM) and PCR. Mosquito collections were made by outdoor landing captures from 21:00–23:00 h. Great differences were found between OM and PCR readings. OM had a sensitivity of 66%, a specificity of 79% and failed to reveal any mixed-infections. Overall prevalence, determined by PCR, was higher in São Tomé (53%) than in Príncipe (35%). It was highest in children below 16 years-old. All four human *Plasmodium* species occurred in São Tomé but *P. ovale* was not detected in Príncipe. The human population was largely asymptomatic. Bednet users had lower prevalence than did non-users. The FOREST form of *Anopheles gambiae s.s.*, identified by PCR and cytogenetics, was the only vector on the islands. The sporozoite rate in São Tomé, assessed by ELISA, was 0.5%. Parasite prevalence and vector densities were positively

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correlated in São Tomé, where malaria transmission must occur predominantly in the more populated coastal areas.

Keywords: Island malaria; Prevalence; São Tomé and Príncipe; *Anopheles gambiae s.s.*; FOREST cytoform; Bednets

1. Introduction

Malaria remains responsible for most hospital admissions and outpatient attendance on the archipelago of São Tomé and Príncipe (STP). The islands are exceptional in that all four species of human malaria are present (Ceita, 1986; Martet et al., 1991).

Measures to control the disease were implemented during the Portuguese administration, but only in the early 1980s, after independence, was a full scale eradication program undertaken. This was based on biannual indoor residual DDT spraying and weekly prophylaxis with chloroquine. As a result, prevalence dropped from 19.2 to 0.6% and incidence, for at least 1 year, fell to zero (Ceita, 1986). The disruption of the spraying programme in 1982, coupled with the introduction of chloroquine-resistant strains of *Plasmodium falciparum*, led to a severe epidemic with many fatalities (Ceita, 1986). Since then, control efforts have been limited. In 1996, a pilot study on the effect of permethrin-impregnated bednets on malaria transmission was initiated in Príncipe and in three areas of São Tomé.

Recent assessment of the epidemiological status of the islands has been restricted to either determination of drug resistance or has been confined to limited areas and age groups (Martet et al., 1991; Loureiro et al., 1996; Ripert et al., 1996; Baptista et al., 1997). In these studies, parasite prevalence was determined by optical microscopy (OM). This technique tends to underestimate rates of low-density infections, particularly of mixed and non-*P. falciparum* species (Looareesuwan et al., 1987; Brown et al., 1992; Snounou et al., 1993a). Carriers of low-density infections could serve as reservoirs from which resurgent parasites and epidemics may arise. The development of highly sensitive and specific polymerase chain reaction (PCR) techniques provide more accurate assessment of parasite prevalence. In a recent study, the majority of travellers returning with malaria parasites from São Tomé were found, by PCR, to be infected with *Plasmodium ovale* or *Plasmodium vivax* (Snounou et al., 1998), otherwise considered the rarest species on the islands.

The vector species composition also remains unclear. In 1946, five species of anophelines were recorded. These included two species of the *Anopheles gambiae* complex and *Anopheles funestus*, all of which harboured malaria sporozoites (Mesquita, 1946). However, the only anophelines found during a survey undertaken in 1986 were *An. gambiae s.l.* and *Anopheles coustani* (Ribeiro et al., 1990). It was not ascertained which members of the *An. gambiae* complex occurred.

Given these considerations, island wide surveys were conducted, as part of an INCO-DC Project, to (i) determine, by PCR, the prevalence and distribution of the different *Plasmodium* species (ii) assess possible differences in infection rates within and between islands and (iii) unequivocally identify the vector. Results are discussed in relation to potential control measures that might be adopted.

2. Material and methods

2.1. Description of the islands

The Democratic Republic STP consists of two main volcanic islands, located in the Gulf of Guinea (West Africa), circa 220 km off the coast of Gabon (Fig. 1). The islands are mountainous, particularly in the south.

The climate is equatorial with average annual temperature of 25°C and average relative humidity above 80% (Ceita, 1986; Denny and Ray, 1989). A cool dry season (gravana) from June–August follows the main rains and there is a second dry period in December. In São Tomé island, average annual rainfall varies between

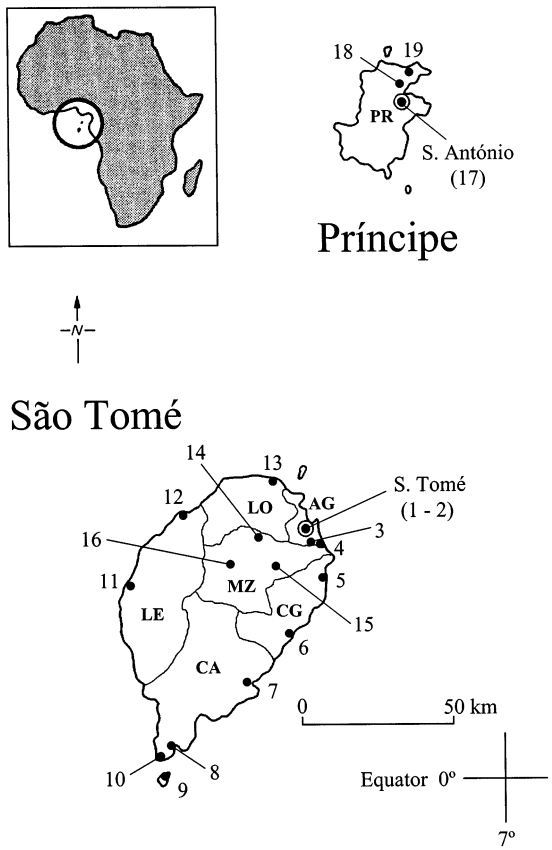

Fig. 1. Map of São Tomé and Príncipe islands showing collection sites. ●, Collection site. ◉, Main city (site numbers in brackets).

District	*N*	Site	Altitude (m)	Settlement	Bednet use (%)
AG (Água Grande)	1	Riboque	0–50	City suburb	18.5
	2	Atrás Cadeia	0–20	City suburb	8.0
	3	S. Gabriel	20–40	Village	69.6
	4	Pantufo	0–20	Village	0.0
CG (Cantagalo)	5	Almoxarife	0–20	Village	14.3
	6	Ribeira Afonso	0–20	Town	37.0
CA (Caué)	7	Angolares	80–100	Town	46.2
	8	Vila Malanza	0–20	Village	0.0
	9	Rolas	0–20	Roça	8.9
	10	Porto Alegre	0–30	Roça	0.0
LE (Lembá)	11	S. Catarina	0–20	Village	0.0
	12	Neves	0–20	Town	14.3
LO (Lobata)	13	Micoló	0–20	Village	41.2
MZ (Me-zoxi)	14	Madalena	260–280	Town	0.0
	15	Trindade	280–300	Town	0.0
	16	Monte Café	700–720	Roça	0.0
PR (Príncipe)	17	Rua Trabalhadores	0–10	City suburb	60.0
	18	Água Doutor	140–150	Village	48.1
	19	Praia Burras	0–10	Village	100.0

Fig. 1. (*Continued*)

1000 mm^3 (north) and 7000 mm^3 (south) and, in Príncipe, between 2500 and 5000 mm^3.

São Tomé (836 km^2) is divided into six administrative districts and has c.a. 140 000 inhabitants, (Fig. 1). Over 60% of the population of São Tomé lives within 10 km of the capital city (São Tomé). The smaller (113 km^2) island of Príncipe, administratively autonomous, has c.a. 7000 people. There is a single town (S. António) with about 2000 inhabitants.

Apart from the capitals, the remainder of the population lives either in (i) towns, with c.a. 2000–5000 inhabitants, usually spread along the main roads; (ii) small villages, usually more isolated or (iii) former colonial plantations (Roças). The south-western part of São Tomé and the southern part of Príncipe are almost uninhabited. In both islands, houses are mainly built with wood walls and zinc roofs and raised on stilts. In the centre of the cities and towns and in the Roças, brick and cement houses prevail. Agriculture and fishing are the main economic activities.

2.2. Collection sites

Parasitological and entomological cross-sectional surveys were carried out between April and June 1997, in 16 localities of São Tomé and in three localities of Príncipe (Fig. 1). Localities were chosen according to their geographical position and to the type of settlement they represented (Fig. 1). Two sites (6 and 13) were from the pilot bednet intervention areas.

2.3. Parasitological survey

Blood samples were taken from the inhabitants of an average of six houses per site in São Tomé, and four houses per site in Príncipe. Houses were selected to have an uniform spatial distribution within each locality. Informed verbal consent was obtained from residents who answered a short questionnaire, which included information on the use of bednets. Parents responded on behalf of infants. Axillary temperature was determined and peripheral blood collected by finger prick. Thick and thin smears were prepared and individual blood spots, on Whatman No. 4 filter paper, obtained. Filter paper samples were dried over silica gel and kept at room temperature in individual plastic bags until further processing by PCR.

Optical microscopy (OM) readings were performed according to Centro Nacional de Endemias (CNE) diagnostic protocols. Slides were Giemsa-stained and parasite numbers per 200 leukocytes determined in thick smears. Parasite density per μl was estimated by the parasite: leukocyte ratio based on 8000 leukocytes/μl. All positive cases by OM received treatment with chloroquine, according to CNE specifications.

The PCR for *Plasmodium* species identification was performed from blood samples in filter paper. After phenol-chloroform DNA extraction, species-specific regions of the small subunit ribosomal RNA genes were amplified by nested-PCR (Snounou et al., 1993b).

2.4. Entomological survey

Relative densities of man biting mosquitoes were determined by outdoor (peridomiciliary) landing captures from 21:00 to 23:00 h in each of the 19 localities studied. Such sampling scheme was chosen due to the reported exophagy and exophily of *An. gambiae s.l.* in STP (Ribeiro et al., 1992) with a biting cycle that reaches his maximum before midnight (Charlwood et al., unpublished).

After morphological identification and counting, samples of *An. gambiae* complex mosquitoes were kept dry over silica gel for sporozoite detection, by ELISA, and species identification, by PCR.

Detection of *P. falciparum* circumsporozoite protein in individual mosquitoes was performed using a two-site ELISA with monoclonal antibodies as previously described (Burkot et al., 1984; Habluetzel et al., 1989). PCR was carried out after DNA extraction from individual mosquitoes (Collins et al., 1988). Amplification of species-specific restriction fragment length polymorphisms of the intergenic spacer regions of the ribosomal DNA, was performed using primers UN (universal), GA (*An. gambiae s.s.*-specific) and ME (*Anopheles melas*/*Anopheles merus*-specific) (Scott et al., 1993).

A sub-sample of females was blood fed and allowed to become half-gravid. These were killed and preserved in Carnoy's (1 part glacial acetic acid: 3 parts absolute ethanol) for subsequent cytogenetic analysis of polytene chromosomes from the ovarian nurse cells, as described by Coluzzi (1968) and Hunt (1973). Chromosomal inversions were scored following the nomenclature of Coluzzi et al. (1979).

2.5. Statistical analysis

Statistical analysis was performed using SPSS 9.0 for Windows® (SPSS, Inc.). Differences between groups were assessed using Pearson's χ^2. Whenever 25% of expected counts were less than 5, significance was determined by Fisher's exact test (2×2 contingency tables) or by the Likelihood Ratio (LR). Within districts, mosquito densities were estimated as the geometric mean of the number of females per man per hour (f/m per h), collected in each locality. Significance of the association between prevalence and mosquito density, in each locality of São Tomé island, was assessed by Spearman's rho non-parametric coefficient (Siegel and Castellan, 1988).

Table 1
Parasite formulae (%) from the islands of São Tomé and Príncipe, determined by optical microscopy (OM) and PCR ($N = 661$)

	P. falciparum	*P. malariae*	*P. vivax*	*P. ovale*
OM	92.4	6.2	0.7	0.7
PCR	82.2	10.2	4.8	2.8

3. Results

3.1. Parasitological data

Five hundred and eighty blood samples from São Tomé and 84 from Príncipe were collected. The age distribution of donors was 0–5 years 24%, 6–15 years 33% and above 15 years 44%. Three hundred and forty-nine of the subjects were female and 315 male. The age structure and sex ratio of inhabitants sampled were similar among districts.

The geometric mean of parasitaemia in Príncipe was 146 parasites/μl (18–8600) and in São Tomé was 273 parasites/μl (16–50 000). Parasitaemias below 100 parasites/μl constituted 39% of all positive slides in São Tomé and 45% in Príncipe.

The results of 661 blood samples analysed by both OM and PCR were compared. One-hundred and fourteen (17%) samples were positive by PCR, but negative by OM. Assuming 100% accuracy for PCR, independent of species, OM had an overall sensitivity (true positives over true positives plus false negatives) of 66% and a specificity (true negatives over true negatives plus false positives) of 79%. Significant differences ($\chi^2 = 23.3$, $df = 3$, $P < 0.001$) were found in parasite formulae determined by OM and PCR (Table 1). PCR showed c.a. 2-fold greater *Plasmodium malariae*, 4-fold greater *P. ovale* and 7-fold greater *P. vivax*. In addition, OM failed to reveal any mixed infections, which constituted 16% of all positives identified by PCR.

Given the differences found between methods and the absence of mixed infections by OM, parasite prevalences were estimated using data from PCR analysis.

Overall malaria prevalence was 53% in São Tomé and 35% in Príncipe. Prevalence did not differ between sexes and was generally higher in under 16 year olds (data not shown).

Prevalence and parasite species distribution according to district are shown in Table 2. In São Tomé, prevalence was highest in the coastal districts. In the inland district (Me-zoxi), prevalence decreased significantly with altitude, from 38% at the lowest locality (site 14) to 4% at the highest ($LR = 10.6$, $df = 2$, $P = 0.005$).

P. falciparum was always the predominant species, followed by *P. malariae*, *P. vivax* and *P.*

Table 2
Malaria prevalence and *Plasmodium* sp. frequencies, determined by PCR, and *An. gambiae* densities, by district and island[a]

District	*N*	PR (%)	Pf (%)	Pm (%)	Pv (%)	Po (%)	FMH
Água Grande	125	56.8	55.2	7.2	4.0	1.6	20.4 (7.5–63.3)
Cantagalo	62	74.2	71.0	4.8	4.8	3.2	44.3 (23.4–84.0)
Caué	154	59.1	56.5	7.8	5.2	1.3	5.8[b] (0.2–63.6)
Lembá	79	64.6	64.6	5.1	1.3	5.1	12.7 (8.0–20.3)
Lobata	85	42.4	40.0	7.1	0.0	0.0	8.2[c]
Me-zoxi	75	18.7	18.7	0.0	0.0	1.3	0.5 (0.3–1.7)
São Tomé (total)	580	53.3	51.6	5.9	2.9	1.9	7.4 (0.2–84.0)
Príncipé	84	34.5	28.6	7.1	2.4	0.0	2.9 (0.5–7.0)

[a] *N*, number of blood samples; PR, prevalence (independent of species); Pf, *P. falciparum*; Pm, *P. malariae*; Pv, *P. vivax*; Po, *P. ovale;* FMH, *An. gambiae* densities as the geometric mean of the number of females caught per man per hour in each locality of the same district. In brackets: range of densities within district.

[b] Including Rolas (site 9) with 0.2 f/m per h. Without that locality, geometric mean FHM rises to 17.9 f/m per h.

[c] Only one locality, absolute value.

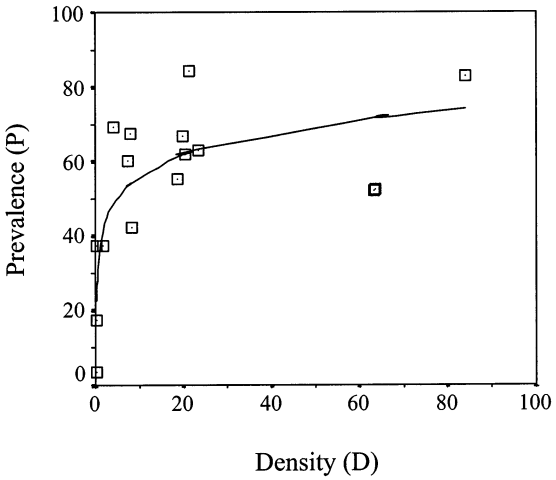

Fig. 2. Malaria prevalence and *An. gambiae* densities per locality in São Tomé island. Prevalence (P): percentage of infected subjects in each locality. Density (D): number of *An. gambiae* females caught per man-hour in each locality. ⊡, Locality; —, logarithmic regression: $(P)_i = 36.34 + 19.61 \log (D)_i + \text{Error}_i$ $(i = 1,\ldots,n)$

ovale, the latter only being found together with *P. falciparum* infections. No *P. ovale* was found in Príncipe. Nine percent of the population sampled in São Tomé and 4% in Príncipe harboured mixed infections. These always contained *P. falciparum*. Two triple infections were detected, both *P. falciparum–P. malariae–P. vivax*.

Only 18 (3%) of 575 subjects had temperatures ≥ 37.5°C. Twelve of these were positive for malaria, all harbouring *P. falciparum* single infections. Eight were children under 6 years old.

Reported bednet use was highest in Príncipe (67%) and, in São Tomé, was confined to the coastal districts (Fig. 1). Prevalence was lower in bednet users than in non-users. Differences were significant in Água Grande ($\chi^2 = 4.5$, d $f = 1$, $P = 0.03$) and Lembá (Fisher's exact test, $P = 0.02$), where 40 and 17% of users and 62 and 69% of non-users harboured malaria parasites, respectively.

3.2. Entomological data

In São Tomé, 2259 *An. gambiae s.l.* females were caught in 108 h of collection. In addition, five *An. coustani* were collected from sites 10 and 11. In Príncipe, 112 *An. gambiae s.l.* were caught in 22 h of collection.

PCR identification of 282 *An. gambiae s.l.* females from São Tomé and 39 from Príncipe, revealed *An. gambiae s.s.*. Similarly, 41 specimens from São Tomé and 8 from Príncipe were all *An. gambiae s.s.* by cytogenetic analysis. Identical results were obtained when sample sizes were increased to 414 (PCR) and 445 (cytogenetics), with specimens subsequently collected in various locations of both islands, during 1997–1998.

With the exception of a single specimen collected outdoor-resting in December 1997 (site 1), no paracentric chromosomal inversions were observed. This specimen was heterozygous for a previously unrecorded inversion on chromosomal arm 2R. The remainder cytogenetically-identified *An. gambiae s.s.* were all monomorphic for the standard chromosome-2 arrangement, characteristic of the FOREST chromosomal form (Coluzzi et al., 1985).

Eight (0.5%) of 1696 An. gambiae females from São Tomé were positive for sporozoites by ELISA. Sporozoite analysis was not undertaken with specimens from Príncipe.

An. gambiae densities in Príncipe were generally lower than in coastal São Tomé (Table 2). In the latter island, lowest densities were recorded in localities above 200 m and in the islet Rolas off the southern coast (Table 2).

In São Tomé, malaria prevalence was positively correlated with *An. gambiae* density (Spearman's rho = 0.6, $P = 0.02$; 2-tailed). Since mosquito density did not follow a normal distribution, a logarithmic regression ($R^2 = 0.58$, $P = 0.001$) was used to describe the relationship between parasite prevalence and mosquito density (Fig. 2).

4. Discussion

Malaria prevalence in STP has increased to levels that exceed the ones recorded before the

implementation of the malaria eradication programme in 1980–1982 (Ceita, 1986).

Independent of identification method, *P. falciparum* was the predominant parasite in both islands. However, the prevalence of species other than *P. falciparum* was significantly underestimated by OM. Mixed infections, none of which detected by OM, accounted for 16% of positive cases by PCR. The very high incidence of *P. ovale* in travellers returning from STP (Snounou et al., 1998) remains unexplained. This was the least common species in São Tomé. It was not found in Príncipe, probably due to the relatively small sample size from that island.

In the present study, the great majority of people, even very young children, were asymptomatic and many had low-density infections. This substantiates the idea that in STP, an immune response to *P. falciparum* develops early in life (Ripert et al., 1996). Sub-patent infections are an important component of the epidemiology of malaria. Several studies using PCR have shown that it is an even more chronic infection than previously suspected (Bottius et al., 1996; Roper et al., 1996; Babiker et al., 1998). Furthermore, despite being 10–100 times more sensitive than OM, a number of parasite positive people may still have remained undetected, due to technical constraints of PCR (Arnot, 1998; Farnert et al., 1999).

The FOREST cytoform of *An. gambiae s.s.* was the only member of the *An. gambiae* complex found. It is almost certainly the only malaria vector of the islands. This chromosomal form typically occurs in rain forest and humid savanna areas (Coluzzi et al., 1985). It is characterised by having chromosome-2 monomorphic standard or few inversions (mostly 2Rb and 2La) at very low frequencies (Petrarca et al., 1987).

Differences in sporozoite rates between districts were not investigated. Nevertheless, the rate of 0.5% fell within the range of those observed from monthly samples collected during 1997–1998, in São Tomé (sites 1 and 5) and Príncipe (sites 17 and 19). In these collections, consistent sporozoite rates of 0.6 and 0.3% were determined for each island, respectively (Charlwood et al., unpublished). Such rates are considerably lower than those recorded from most other areas of Africa (Gillies and de Meillon, 1968; Gillies and Coetzee, 1987). In particular, sporozoite rates were much lower than those from the neighbouring island of Bioko (c.a. 10%) (Molina et al., 1993), and point to a differing transmission pattern on the different islands of the Gulf of Guinea.

Overall, prevalence, parasite density and *An. gambiae* density were lower in Príncipe than in coastal São Tomé. The survey on Príncipe was the last to be performed, taking place during the transition from the wet to the cool dry season (gravana). Although this might have influenced vector density, it is unlikely to have affected parasite prevalences. Collections conducted, in the rainy season of 1998 (April), resulted in similar or greater mosquito densities than those recorded from São Tomé. Hence, lower mosquito numbers may not explain the lower endemicity found in Príncipe. These differences could, however, reflect the greater use of bednets in the sampled population of Príncipe. Although *An. gambiae* in STP is largely exophilic and exophagic (Ribeiro et al., 1992), significant differences in malaria prevalences were detected between bednet users and non-users. Prevalence is the least sensitive indicator of any anti-malarial intervention. Thus, although not measured, it is likely that incidence and mortality would also be reduced by the use of bednets. Their use on the islands should be encouraged.

In São Tomé island, malaria is largely confined to the coastal fringe. At altitudes above 200 m, both parasite prevalence and vector densities showed a marked decline. This is well below the altitude at which parasite and vector can survive (Gillies and de Meillon, 1968; Lindsay and Martens, 1998). The heavy rainfall on the steep sided slopes probably flushes larvae from breeding sites and may be responsible for low mosquito densities in these inland areas.

Prevalence was positively correlated with vector density, in São Tomé. If transmission differences predominantly depend on vector density, control measures directed against the vector should have a direct impact on the disease. Differences in vector density influenced the epidemiology of malaria in Dakar (Trape et al., 1992). Elsewhere,

however, the proportion of infective bites showed a marked decrease as the biting rates increased (Charlwood et al., 1998), and in some situations higher parasite prevalences may even be associated with lower mosquito densities (Thomson et al., 1994).

Malaria in STP, as everywhere else, is a disease of poverty. There remain serious obstacles to its control. A large population of asymptomatic carriers facilitates the maintenance of parasite reservoirs and the presence of chloroquine resistant strains makes treatment difficult. Exophily may also pose problems in reducing the vector population using strictly intradomiciliary measures. Nevertheless, the presence of a single vector species, with almost non-existent chromosomal variation, simplifies the epidemiology when compared to multi-species vectorial systems often found in the continent. The immigration of potential vectors from the mainland is also likely to be limited. Given these considerations, perhaps it is not too much to hope that malaria on São Tomé and Principe could be controlled.

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References

Arnot, D., 1998. Unstable malaria in Sudan: the influence of the dry season: clone multiplicity of *Plasmodium falciparum* infections in individuals exposed to variable levels of disease transmission. Trans. R. Soc. Trop. Med. Hyg. 92, 580–585.

Babiker, H.A., Abdel-Muhsin, A.-M.A., Randford-Cartwright, L.C., Satti, G., Walliker, D., 1998. Characteristics of *Plasmodium falciparum* parasites that survive the lengthy dry season in eastern Sudan where malaria transmission is markedly seasonal. Am. J. Trop. Med. Hyg. 59, 582–590.

Baptista, J.L., Neves, I., D'Alessandro, U., Hendrix, L., Wéry, M., 1997. *Plasmodium falciparum* chloroquine and quinine sensitivity in asymptomatic and symptomatic children in São Tomé island. Trop. Med. Int. Health 2, 582–588.

Bottius, E., Guanzirolli, A., Trape, J.-F., Rogier, C., Konate, L., Druilhe, P., 1996. Malaria: even more chronic in nature than previously thought; evidence for subpatent parasitaemia detectable by the polymerase chain reaction. Trans. R. Soc. Trop. Med. Hyg. 90, 15–19.

Brown, A.E., Kain, K.C., Pipithkul, J., Webster, H.K., 1992. Demonstration by the polymerase chain reaction of mixed *Plasmodium falciparum* and *P. vivax* infections undetected by conventional microscopy. Trans. R. Soc. Trop. Med. Hyg. 86, 609–612.

Burkot, T.R., Zavala, F., Gwadz, R.W., Collins, F.H., Nussenzweig, R.S., Roberts, D.R., 1984. Identification of malaria-infected mosquitoes by a two-site enzyme-linked immunosorbent assay. Am. J. Trop. Med. Hyg. 33, 227–231.

Ceita, J.G.V., 1986. Malaria in São Tomé and Príncipe. In: Buck, A.A. (Ed.), Proceedings of the Conference on Malaria in Africa. American Institute of Biological Sciences, Washington DC, pp. 142–155.

Charlwood, J.D., Smith, T., Lyimo, E., et al., 1998. Incidence of *Plasmodium falciparum* infection in infants in relation to exposure to sporozoite-infected anophelines. Am. J. Trop. Med. Hyg. 59, 243–251.

Collins, F.H., Mehaffey, P.C., Rasmussen, M.O., Brandling-Bennett, A.D., Odera, J.S., Finnerty, V., 1988. Comparison of DNA-probe and isoenzyme methods for differentiating *Anopheles gambiae* and *Anopheles arabiensis* (Diptera: Culicidae). J. Med. Entomol. 25, 116–120.

Coluzzi, M., 1968. Cromosomi politenici delle cellule nutrici ovariche nel complesso *gambiae* del genere *Anopheles*. Parassitologia 10, 179–184.

Coluzzi, M., Petrarca, V., Di Deco, M.A., 1985. Chromosomal inversion intergradation an incipient speciation in *Anopheles gambiae*. Boll. Zool. 52, 45–63.

Coluzzi, M., Sabatini, A., Petrarca, V., Di Deco, M.A., 1979. Chromosomal differentiation and adaptation to human environments in the *Anopheles gambiae* complex. Trans. R. Soc. Trop. Med. Hyg. 73, 483–497.

Denny, L.M., Ray, D.I., 1989. São Tomé and Príncipe. Economics, politics and society. Marxist Regime Series. Printer Publishers, London, pp. 150.

Farnert, A., Arez, A.P., Correia, A.T., Bjorkman, A., Snounou, G., Rosário, V.E., 1999. Sampling and storage of blood and the detection of malaria parasites by polymerase chain reaction. Trans. R. Soc. Trop. Med. Hyg. 9, 50–53.

Gillies, M.T., Coetzee, M., 1987. A supplement to the Anophelinae of Africa south of the Sahara. Publications of the South African Medical Institute for Medical Research No. 55, Johannesburg, pp. 143.

Gillies, M.T., de Meillon, B., 1968. The Anophelinae of Africa south of the Sahara. Publications of the South African Medical Institute for Medical Research No. 54, Johannesburg, pp. 343.

Habluetzel, A., Esposito, F., Lombardi, S., 1989. Immunotechniques for epidemiology of malaria: appropriate tools for integration of primary health care with malaria research and control. Trans. R. Soc. Trop. Med. Hyg. 83, 15–19.

Hunt, R.H., 1973. A cytological technique for the study of *Anopheles gambiae* complex. Parassitologia 15, 137–139.

Lindsay, S.W., Martens, W.J.M., 1998. Malaria in the African highlands: past, present and future. Bull. World Health Organ. 76, 33–45.

Looareesuwan, S., White, N.J., Chittamas, S., Bunnag, D., Harinasuta, T., 1987. High rate of *Plasmodium vivax* relapse following treatment of *falciparum* malaria in Thailand. Lancet 2, 1052–1054.

Loureiro, L.F., Cesário, A.M., Franco, A.S., Rosário, V.E., 1996. Malaria in São Tomé and Príncipe: prevalence and drug-susceptibility. Ann. Trop. Med. Parasitol. 90, 223–224.

Martet, G., Conceição, S., Cordoliani, G., et al., 1991. Le paludisme en République de São Tomé et Príncipe: évaluation épidémiologique et chimiorésistance de *P. falciparum*. Bull. Soc. Pathol. Exot. 84, 273–280.

Mesquita, V.H.B., 1946. Contribuição para o estudo do sezonismo em. S. Tomé. Bol. Ger. Col. 22, 13–66.

Molina, R., Benito, A., Roche, J., et al., 1993. Baseline entomological data for a pilot malaria control programme in Equatorial Guinea. J. Med. Entomol. 30, 622–624.

Petrarca, V., Vercruysse, J., Coluzzi, M., 1987. Observation on the *Anopheles gambiae* complex in Senegal River Basin, West Africa. Med. Vet. Entomol. 1, 303–312.

Ribeiro, H., Ramos, H.C., Janz, J.G., 1992. The feeding and resting behaviour of *Anopheles gambiae* on the islands of São Tomé and Príncipe, West Africa. Bull. Soc. Vector Ecol. 17, 25–27.

Ribeiro, H., Ramos, H.C., Pires, C.A., 1990. Sobre os vectores da malária em São Tomé e Príncipe. Garcia de Horta Ser. Zool. 15, 135–152.

Ripert, C., Neves, I., Appriou, M., et al., 1996. Épidémiologie de certaines endémies parasitaires dans la ville de Guadalupe (République de São Tomé et Príncipe) II: autres endémies parasitaires. Bull. Soc. Pathol. Exot. 89, 259–261.

Roper, C., Elhassan, I.M., Hviid, L., et al., 1996. 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. Am. J. Trop. Med. Hyg. 54, 325–331.

Scott, J.A., Brogdon, W.G., Collins, F.H., 1993. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. Am. J. Trop. Med Hyg. 49, 520–529.

Siegel, S., Castellan, N.J. Jr, 1988. Non-Parametric Systems for the Behavioural Sciences, 2nd. McGraw-Hill, Singapore, pp. 399.

Snounou, G., Pinheiro, L., Antunes, A.M., Ferreira, C., Rosário, V.E., 1998. Non-immune patients in the Democratic Republic of São Tomé e Príncipe reveal a high level of transmission of *P. ovale* and *P. vivax* despite low frequency in immune patients. Acta Trop. 70, 197–203.

Snounou, G., Pinheiro, L., Gonçalves, A., et al., 1993a. The importance of sensitive detection of malaria parasites in the human and insect hosts in epidemiological studies, as shown by the analysis of field samples from Guinea Bissau. Trans. R. Soc. Trop. Med. Hyg. 87, 649–653.

Snounou, G., Viriyakosol, S., Zhu, X.P., et al., 1993b. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol. Biochem. Parasitol. 61, 315–320.

Thomson, M.C., D'Alessandro, U., Bennett, S., et al., 1994. Malaria prevalence is inversely related to vector density in The Gambia, West Africa. Trans. R. Soc. Trop. Med. Hyg. 88, 638–643.

Trape, J.-F., Lefebvre-Zante, E., Legros, F., et al., 1992. Vector density gradients and the epidemiology of urban malaria in Dakar, Senegal. Am. J. Trop. Med. Hyg. 47, 181–189.