Asymptomatic *Plasmodium falciparum* malaria and gametocyte carriage are common in Coastal Kenya

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SUMMARY

Adequate malaria diagnosis and treatment remain a major problem in rural sub Saharan Africa. Molecular parasite detection has shown that submicroscopic *Plasmodium falciparum* asexual and gametocytes are common in patients, and can infect mosquitoes in low endemic areas. The significance of the infectious reservoir of malaria in the general population remains unknown. In this study we investigated submicroscopic asexual parasitaemia and gametocytaemia in inhabitants of areas of hypo-endemic and seasonal malaria in which no molecular analysis on malaria survey has been done before.

Cross-sectional stuies during the staging of two clinical trials clinical trial in Mokowe and Lamu, Coastal Kenya were conducted in the dry and wet seasons of 2010. Finger prick blood samples used to determine the prevalence of *Plasmodium falciparum* parasites by microscopy, Fluorescence In-situ hybridization (FISH), rapid diagnostic test and real time nucleic acid sequence-based amplification (QT-NASBA).

A total of 450 individuals participated in the surveys of whom, 2.7% had microscopically confirmed asexual parasites while 2.4% had gametocytes. In contrast, FISH revealed that 8.9% (29/327) and QT-NASBA 24.6% (59/240) of the individuals harbored asexual parasites and 29.2% (70/240) gametocytes. There were a few cases of mixed infection with *Plasmodium malariae*, 1.8% (8/ 450) by microscopy and 4% (13/327) by FISH. No age dependency or seasonality was observed in the submicroscopic parasite carriage.

In conclusion, molecular detection techniques disclose that carriage of submicroscopic asexual parasite and gametocyte is relatively common in these low transmission areas and that submicroscopic gametocytaemia is likely to be responsible for maintaining malaria transmission in the study area.

Key words: submicroscopic, gametocytaemia, FISH, rapid diagnostic test, QT-NASBA

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Introduction

Gametocytes are the sexual stage parasites that are responsible for transmission of malaria from human to mosquito. Successful mosquito infection requires the ingestion of mature gametocytes by mosquitoes during a blood meal. The production of gametocytes is essential for maintaining malaria transmission, particularly in areas of low or seasonal malaria transmission. This is important where levels of human-vector contact are unpredicted and a consistent production of gametocytes appears necessary for parasite survival [1, 15].

Gametocyte carriage is most commonly assessed microscopically and is related to malaria transmission intensity. In high endemic areas most microscopic gametocyte carriers are children, while this negative age dependency disappears as transmission intensity decreases as in [16] and [23].

Microscopically confirmed gametocytes carriage is generally uncommon in low transmission areas, especially in the dry season [1, 2 and 13] although gametocyte carriers are vital in re-initiating malaria transmission in the subsequent transmission season. During the dry season, asexual parasite carriage can persist below the microscopic threshold [3] and this may also be true for gametocyte carriage.

Molecular gametocyte detection techniques have addressed submicroscopic *Plasmodium falciparum* gametocytaemia in microscopically confirmed asexual parasite carriers [1, 8, 15 and 20]. Studies using either reverse transcriptase PCR (RT-PCR) or quantitative nucleic acid sequence-based amplification (QT-NASBA) have revealed that submicroscopic gametocytaemia is common in symptomatic parasite carriers and can persist for several months [1, 8, 15 and 20] in asymptomatic carriers. The prevalence and importance of submicroscopic gametocytaemia has however yet to be investigated in the general population of neglected areas for malaria control. In this study we address this issue by determining the prevalence of submicroscopic asexual parasites and gametocytes in inhabitants of an area of low and seasonal malaria endemicity in Coastal Kenya.

Materials and Methods

Study sites

This study was conducted in Mokowe, the mainland to the Indian Ocean and neighboring Lamu Island. Malaria transmission in the area is largely restricted to the long rainy season (May-July) and an unpredictable short rainy season (October- November). The remainder of the year is dry and hot and transmission is practically absent. The average annual rainfall is 615 mm (10 years average) and the entomological inoculation rate has not been estimated in these villages (unpublished paper). Villagers predominantly belong to Bajunis, Ormas, Arabs and some few Pokomo and Kikuyu ethnic groups, and are mostly involved in self-subsistence farming and fishing.

Study design

Two cross sectional surveys were conducted during the dry season (March) and wet season (July- August) in the year 2010 at the mainland of Mokowe, targeting a minimum of 200 participants per survey. Families were selected using village censuses and computer randomized tables. A central point in each village was identified and villagers attended the survey on a first come first serve.

Ethical considerations

All villagers who were invited for the survey were given detailed information about the study aim and procedures before they were asked to sign individual informed consent. Parents/ guardians were asked to sign on behalf of children. The study protocol was approved by the Scientific Steering Committee (SSC) and Ethical Review Committee (ERC) of Kenya Medical Research Institute [Protocol No, 1556 (b)].

Data collection and processing

Information on demographic, anthropometric and general heath indicators were collected by means of a questionnaire. Each participant was examined by a qualified clinical officer for signs of anemia, fever and splenomegally. Subjects were recruited at the hospital of King Fahad, Mokowe clinic and came from within a radius10 km. Children from 6 months-15 years were included when they had either a temperature of ≥37.5 °C or a history of fever in the last 24h and had a mono-infection with *P. falciparum* with an asexual parasite density of 1,000-200,000 parasite/µL or mixed infection. Exclusion criteria were haemoglobin levels lower than 5 g/dl, presence of other disease which causes febrile conditions, or signs of severe malaria.

Treatment

Recruited children were treated with AL (Coartem®; Novartis Pharma) administered as half a tablet (20 mg of artemether and 120 mg of lumefantrine) per 5 kg of body weight in a 6-dose regimen (at enrolment and 8, 20, 32, 44 and 56 hours [±90 minutes] after initiation of treatment). Drugs were given under supervision with local fatty food to facilitate absorption; the first dose was given at the clinic, the remaining doses at home during home visits of trained field workers. Children were observed for 30 minutes after administration of treatment, and a replacement dose was given if vomiting occurred. Repeated vomiting led to exclusion from the study [24].

Sample collection

A single blood sample was obtained by finger prick from each participant and used in the field for hemoglobin (Hb) measurement using a haemocue photometer (Angelholm, Sweden), detection of malaria parasites using a rapid diagnostic test (RDT) detecting *P. falciparum* specific histidine rich protein-2 and the preparation of microscopy and QT-NASBA samples. The RDT and Hb measurement were performed according to the manufacturer's instructions and scored in the field. Those found to have malaria by RDT were given the free treatment with Artemisinin-Lumefantrine (AL) according to the national guidelines. Individuals requiring further attention were treated accordingly and there were no referral cases [24].

Parasite detection by microscopy

Thin and thick blood smears were made and Giemsa stained in the field. Slides were read in the field site and then followed subsequently double read in two different stations by experienced microscopists.

Parasite detection by FISH

For Fluorescence In-Situ Hybridization (FISH) analysis, thin blood smear were made and fixed with methanol in the field. The slides were then reserved well and taken to the lab for the fluorescence microscope view. Prepared kit was used for *Plasmodium* Genus ID-FISH test developed by ID-FISH technology Inc. (San Antonio, California) for detection of all *Plasmodium* species (unpublished

paper). This kit comprised of the fluorescent labeled probe, the hybridization buffer, three buffers (Pretreatment buffer, Wash Buffer I and Wash Buffer II) and Evans Blue Counter Stain.

Parasite detection by QT-NASBA

Blood from finger prick of about 50µl was collected in EDTA tubes for Quantitative Nucleic Acid Sequence Based Amplification (QT-NASBA). *Plasmodium falciparum* parasite detection by QT-NASBA was done as described elsewhere by Schneider [18 and 19]. Nucleic acid was extracted from blood samples as described by Boom [10]. The first part of the RNA extraction was done in the field following the original Guanidine isothiocynate (GuSCN) RNA extraction method [13] until the nucleic acids were bound to silica dioxide particles. The samples were then stored at -20°C and transferred to the laboratory for completion of the extraction and QT-NASBA analysis. QT-NASBA was performed on a Bio-Rad machine having CFX96 Real Time System for 18S rRNA and Pfs25 mRNA. The 18S QT-NASBA detects all circulating parasite stages while the Pfs25 QT-NASBA is gametocyte specific. Although the number of asexual parasites cannot be directly determined by QT-NASBA, it can be estimated by subtracting the concentration of gametocytes in the Pfs25 QT-NASBA from the total concentration of parasites in the 18S QT-NASBA. Nuclisense Basic kits were used for amplification according to the manufacturers' manual (**table 1**). The reaction is isothermal (41°C) and relies on the simultaneous activity of three enzymes AMV-RT, Rnase H and T7 RNA polymerase. The low temperature and inclusion of the T7 polymerase-promoter in one of the amplification primers ensures that primers only anneal to single stranded target RNA. This prevents amplification of genomic DNA that may be present in the sample and allows for specific detection of living, metabolically active cells and organisms. The amplification process is part of a total system, which includes a nucleic acid isolation procedure, and powerful detection methodology [19].

Table 1: Primers and probes for 18S and 25S *P. falciparum* NASBA

Pf 18 QT-NASBA	Forward primer: 5'-gtcatctttcgaggtgactt-3' (nucleotides 1136-1155)
	Reverse primer: 5'aattctaatacgactcactatagggagaaggaactttctcgcttgcgcgaa-3'(T7 promoter sequence, linker and nucleotides 1216-1235)
	Pf18S molecular beacon: 5'-FAM-cgatcg-gagaaatcaaagtctttggg-cgatcg-DABSYL-3' (molecular beacon stem of 6 paired nucleotides and nucleotides 1182-1201).
Pf 25 QT-NASBA	Forward primer ECL NASBA: 204-227 sense 5'-GACTGTAAATAAACCATGTGGAGA-3'
	Reverse primer ECL NASBA: T7+linker+338-359 antisense 5'-AATTCTAATACGACTCACTATAGGGAGAAGG-CATTTACCGTTACCACAAGTTA-3'
	Pfs25 molecular beacon 5'-Texas_Red-cgatcg-cccgtttcatacgcttgtaa-cgatcg-DABSYL-3'

The number of gametocytes was calculated in relation to standard gametocyte stage V dilution series [17] using the time point of amplification at which the fluorescence detecting target amplicons exceeded the mean fluorescence of three negative controls +20 standard deviations. Parasite detection by QT-NASBA was done for150-180 individuals from three age groups (< 5 years, ≥5 to 15 years and ≥ 15 years). Samples were randomly selected within age-strata using computer generated randomization tables [19].

Data analysis

All data was double entered in a created computer access database and imported in strata 10.0 after validation. Age was categorized into < 5 years, ≥5 to 15 years and ≥ 15 years; hemoglobin status in severe (5g Hb/dl), moderate (<8g/dl), and mild anaemia (<11g/dl). Proportions were compared between groups with the x^2- test or Fisher's exact test; means were compared with one-way ANOVA, student t-test or Wilcox on Rank-sum test, where appropriate. The correlation between normally distributed continuous variable was quantified using the Pearson's correlation coefficient, the relation between gametocyte prevalence and asexual parasite density by logistic regression models. Parasite density was analyzed on a log-scale and geometric mean densities per ml were calculated for parasite carriers with interquartile ranges (IQR).

Results

In total, 450 individuals participated in the cross-sectional surveys, 156 in the dry season and 294 in the wet season (**table 2**). The prevalence of moderate or severe anaemia (Hb<8g/dl was 5.8% (26.1/ 450) in the general population but was higher in children under five years of age, 17.2% (20/115) (x^2= 97.70, p< 0.001).

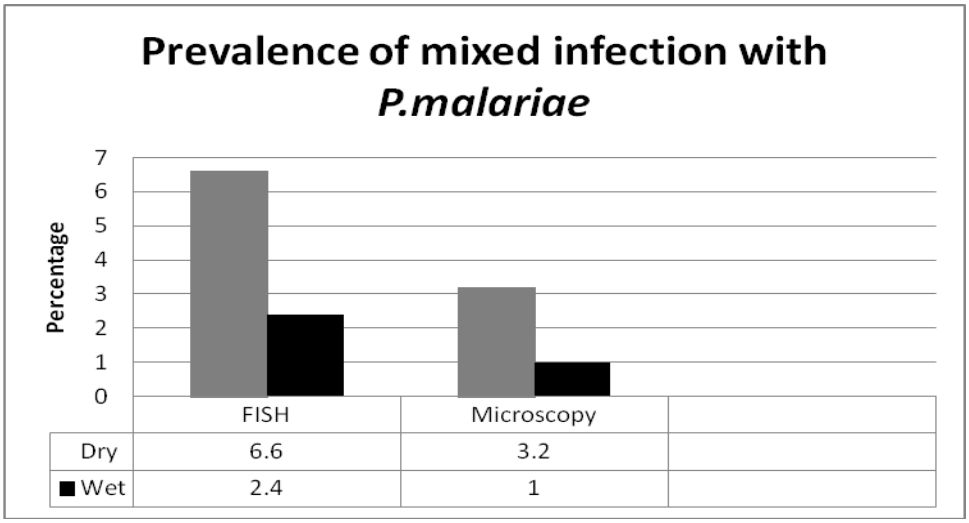
Table 2: Characteristics of the study populations

N		450
	Dry season, n	156
	Wet season, n	294
Age (Years), % (n)		
	<5	25.5 (115)
	5-15	40.3 (181)
	>15	34.2 (154)
Tribe, % (n)		
	Cajuns	52.5 (236)
	Orcas	20.7 (93)
	Arabs	15.3 (69)
	Others	11.5 (52)
Reported fever in previous two weeks, % (n)		15.7 (71)

The rapid diagnostic test (RDT) detected parasites in 2.7% (4/156) of the individual in the dry season and 1.5% (4/ 294) in the wet season (x^2=4.23, p=0.025). Asexual parasite prevalence as detected by microscopy was 2.7% (12/ 450) as total in dry and wet season (**table3**). There was a statistically significant positive association between the presence of parasites by RDT and microscopy (x^2= 254.20, p< 0.001).

In contrast due to the low prevalence of microscopically confirmed parasite carriers, the FISH detected parasites in 8.9% (29/327) of the individuals with mono-infection with *P. falciparum* and 4% (13/327) having mixed infections with *P. malariae* (figure 1).

Figure 1: Prevalence of mixed infection



With the QT-NASBA, the 18s detected parasites in 24.6% (59/240) of the individuals. Parasite prevalence by 18S QT-NASBA was significantly moderate in the dry season (x^2= 4.02 p= 0.02). For those individuals who were 18S positive and Pfs25 QT-NASBA negative, 18S QT-NASBA parasite concentration was assumed to equal asexual parasite density. In this study, the prevalence of 18S QT-NASBA parasite concentration was low compared to Pfs25 QT-NASBA parasite concentration, and the presence of asexual parasite was approved by following with the FISH technique. Gametocytes were detected by microscopy in 2.4% (11/ 450) of the slides, the Pfs25 QT-NASBA detected gametocytes in 29.2% (70/450) of the individuals, independent of season (**table 3**). The geometric mean gametocyte density by Pfs25 QT-NASBA was 108.2 gametocyte/ml (IQR 20.0-1045.0). There was no strong correlation between the estimated density of asexual parasites by 18S QT-NASBA and Pfs25 QT-NASBA gametocyte prevalence (R^2 0.50, p= 0.007). Parasites densities of those individuals whose samples were tested by QT-NASBA, FISH and microscopy (n=300) are presented in the **table 3**. The vast majorities of parasite carriers detected by 18S QT-NASBA harbored parasites at a low density and were not detected by microscopy.

Table 3: Parasite prevalence by different detection technique

Parasite prevalence, % (95% CI)						
Season	Rapid Test	Microscopy		QT-NASBA		FISH (*Puff*)
		Asexual	Gametocyte	Asexual	Gametocyte	
Dry	2.7(4/156)	5.8 (9/156)	1.9 (3/156)	28.1 (27/96)	35.1 (34/96)	17.2(21/121)
Wet	1.5(4/294)	1.0 (3/294)	2.7 (8/294)	22.2(32/144)	25.3(36/144)	3.9 (8/206)
Total	1.8(8/450)	2.7(12/450)	2.4(59/240)	24.6(59/240)	29.2(70/240)	8.9 (29/327)

95% CI= 95% interval;

Discussion

A first indication of a reduced sensitivity to ACTs in Africa was recently reported in coastal Kenya where the parasite clearance time after Artemether-lumefantrine (AL) and Dihydroartemisinin-piperaquine (DHA-PPQ) increased between 2005-2006 and 2007-2008 [6]. In this study we investigated submicroscopic *P. falciparum* parasitaemia in a cross section of the general population of a low transmission area in coastal Kenya.While microscopically confirmed carriage of asexual parasites and gametocytes was very low, the QT-NASBA revealed that 24.6% of the individuals carried asexual parasites and 29.2% gametocytes.

The high prevalence of submicroscopic parasitaemia especially with gametocyte was not related to age or season. The absence of a clear seasonal difference in parasite prevalence is most likely due to the limited rainfall in the year we conducted at the study site. The total rainfall in 2010 was only 29% of the 10 year average and was particularly poor in the months of April and May. Although asymptomatic parasite carriage is well known in areas with perennial transmission, it is remarkable in an area of low and seasonal malaria transmission. The prevalence of submicroscopic parasitaemia was previously determined in areas of low malaria transmission intensity although results are not directly comparable with ours because of differences in malaria transmission patterns [13] and the selection of individual [10]. Only 6- 15% of individuals from an epidemic-prone area of low transmission in Kenya harbored parasites by PCR in the dry or wet season [13] and 40-50% of treated symptomatic patients retained persisting parasitaemia by PCR during the dry season in an area of unstable malaria in Sudan[10].

Our findings suggest that even in an area of very low transmission intensity, a substantial part of the population is capable of controlling infections in such a way that parasite densities remain extremely low. These symptomatic parasite carriers can be expected to become gametocytaemic at some point during their infection if a regular number of asexual parasites are committed to the sexual pathway, as is suggested by mathematical models [4]. Indeed, we observed that a relatively large proportion of parasite carriers harbored gametocytes at submicroscopic densities at the time of sampling. Our gametocyte prevalence of 29.2% measured by QT-NASBA compared with 12-52% in longitudinal followed individuals after antimalarial

treatment in an area of unstable malaria in Sudan [1]. Sequestration of gametocytes in sub dermal capillaries [16] suggests that some gametocytes carriers may have remained undetected by examining finger prick blood samples. The true proportion of gametocyte carriers in our population may therefore be even larger than detected by Pfs25 QT-NASBA.

The relevance of these high-density gametocyte infections to the overall infectious reservoir of malaria needs some consideration. It is generally accepted that the presence of gametocytes does not always render the human host infectiousness to mosquitoes. Infectivity is influenced by several factors, including immune response, gametocyte fitness and density [21-23]. Thus, rather than gametocyte density per se, malaria transmission potential in mathematical models is often defined as the probability that a mosquito blood meal contains the threshold density of at least one gametocyte of each sex [12]. This threshold is 1 gametocyte per µl assuming an average mosquito blood meal size of 2µl. In this study, only 30% of the gametocyte carriers harbored gametocytes at or above this theoretical threshold density. However, data are accumulating to suggest that this threshold density is too high; submicroscopic densities are infectious to mosquitoes [7, 9] and this has recently been shown using the Pfs25 QT-NASBA to detect gametocytes [8]. Using this technique, mosquito infection can be successful at densities below 1 gametocyte/µl, for both natural infections and cultured gametocytes [11]. Although the proportion of mosquitoes infected is lower for carriers with submicroscopic gametocytaemia [15, 22] they are present in substantial numbers and their contribution to malaria transmission can be considerable [8, 9, 11].

The use of molecular techniques provides more precise measures of parasite prevalence. These measures suggest more asymptomatic infections and longer periods of carriage of asexual parasites, which will presumably affect the generation of effective immunity and a threat of eruption of malaria cases in these regions. In such situations, a vibrant malaria surveillance program would provide more apt reporting on rising incidence of cases in these regions.

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