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Persistent detection of Plasmodium falciparum, P. malariae, P. ovale curtisi and P. ovale wallikeri after ACT treatment of asymptomatic Ghanaian school-children

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Abstract

Two hundred and seventy four asymptomatic Ghanaian school-children aged 5 to 17 years were screened for malaria parasites by examination of blood films. One hundred and fifty five microscopically-positive individuals were treated with dihydroartemisinin-piperaquine and followed for 3 weeks. Retrospective species-specific PCR of all 274 screened samples identified an additional 60 children with sub-patent parasitaemia, and a substantial proportion of co-infections with Plasmodium malariae, Plasmodium ovale curtisi and Plasmodium ovale wallikeri. One hundred individuals harboured at least one non-falciparum parasite species. Using standard double-read microscopy, the 21-day efficacy of treatment against Plasmodium falciparum was 91.4% among the 117 children seen at all 5 visits. Using nested PCR to test 152 visit 5 blood samples, 22 were found to be parasite-positive. Twenty individuals harboured P. falciparum, four harboured P. ovale spp. and two P. malariae, with four of these 22 isolates being mixed species infections. The persistent detection of low density Plasmodium sp. infections following antimalarial treatment suggests these may be a hitherto unrecognised obstacle to malaria elimination.

1. Introduction

Studies of malaria in sub-Saharan Africa have been focused on a single parasite species, Plasmodium falciparum, because of its predominance among clinical cases, and its association with severe disease and mortality. Three other species are widespread in Africa but generally thought to be uncommon among clinical malaria cases: Plasmodium malariae, Plasmodium ovale curtisi and Plasmodium ovale wallikeri (Collins and Jeffery, 2007; Sutherland et al., 2010). However recent cross-sectional population-based studies in Malawi, Uganda, Equatorial Guinea and Angola, using PCR for parasite detection and species discrimination, have shown that these three species are found in between 1% and 17% of tested individuals, regardless of whether malaria symptoms were present (Bruce et al., 2008; Oguike et al., 2011; Fançony et al., 2012). In each of these studies, the majority of these occurrences were as co-infections with P. falciparum, and we therefore surmise that, in Africa, P. malariae and P. ovale spp. will frequently encounter control measures and case management strategies designed specifically for falciparum malaria.

Infections with non-falciparum malaria parasites have been adequately treated with chloroquine for many decades (Lalloo et al., 2007). However, in most African settings, species-level diagnosis is not available for people suffering from malaria (or suspected malaria), and so virtually all patients are treated for P. falciparum and are thus likely to receive artemisinin-combination therapy (ACT) where these drugs are available. Unfortunately, there are few in vivo data available to indicate how well non-falciparum species respond to ACT. Clinical trials commonly exclude mixed infections at enrolment and molecular characterisation of treated parasite isolates is not commonplace. Partially based on evidence for good efficacy of ACT against Plasmodium vivax in Asia (Kolaczinski et al., 2007; Douglas et al., 2010), it is expected that ACT will be very effective against P. malariae, P. o. curtisi and P. o. wallikeri. Recent evidence from Gabon would support this view (Mombo-Ngoma et al., 2012). As current research is now illuminating the moderately high prevalence of these species across Africa, it is important to assess the response of non-falciparum parasites to commonly used antimalarial drugs, and in particular to ACT.

As part of a study of P. falciparum gametocyte carriage and associated antibody responses, asymptomatic Ghanaian school...
children with parasitaemia were enrolled in a longitudinal study. Microscopy-confirmed *P. falciparum* malaria infections were treated with a full course of the ACT dihydroartemisinin-piperquine (DP). As an ancillary analysis, we tested for the presence of all *Plasmodium* species by PCR, both prior to and 3 weeks after treatment, and estimated drug efficacy against sub-patent parasitaemia.

### 2. Materials & methods

#### 2.1. Study area

The study was conducted during the months of October–December 2010, in Pokukrom in the Ahafo Ano South District of the Ashanti region, which lies in the tropical rainforest ecological zone of Ghana. The mean monthly temperature ranges between 18 and 38 °C and the average rainfall in the neighbouring area of Kintampo is 1250 mm per annum, occurring mainly between May and October each year (Owusu-Agyei et al., 2009). The district is considered endemic for malaria and a parasite carriage rate of 65% has previously been recorded (H. Tagbor, unpublished data). Transmission of malaria in this area is perennial but peaks during the rainy season. Malaria is predominantly caused by *P. falciparum* (Asante et al., 2011) and no published studies have looked at the prevalence of other malaria species in the district. The main vectors are *Anopheles gambiae* and *Anopheles funestus*; no current estimates of entomologic inoculation rate are available for Ahafo Ano District, but in Kintampo this is estimated to be 250 infectious bites per year (Owusu-Agyei et al., 2009). Laboratory support and microscopy facilities were provided at the Kwame Nkrumah University of Science and Technology (KNUST), Department of Biology, Kumasi. The study protocols were approved by the Ghana Health Service Ethics Committee (proposal # GHS-ERC-08/7/10) and the Ethics Committee of the London School of Hygiene and Tropical Medicine (proposal # 5775). Individual informed consent was obtained from the children and their parents/guardians before enrolment. Meetings were also held in the community and schools to explain the objectives of the study and to seek the consent of the community leaders and education authorities.

#### 2.2. Participants and sample collection

Asymptomatic school children of Pokukrom Methodist primary between the ages of 5 and 17 years were screened for asexual malaria parasites in finger-prick peripheral blood. For each sample a rapid immunochromatographic point-of-care test (RDT) for antigenaemia was carried out (Malaria Pf rapid test, Shenyang LTH Technology Development Company, Beijing, China), blood smears made for microscopy, and approximately 10–50 μL of blood spotted directly from the child’s finger onto Whatman grade 3 filter paper (Whatman, Maidstone, UK). Microscope slides were stained with 10% Giemsa. Each daily dose of the fixed-combination was administered undiluted and taken with a full glass of water. All enrolled children were followed up for repeat finger-prick blood samples weekly for 4 weeks after the first blood sample was taken; as treatment was given on the second visit, each child was thus seen three further times after treatment. During this period the participants had access to free anti-malarial treatment and clinical care if required. The longitudinal study was designed to permit testing for plasma antibodies against *P. falciparum* gametocyte antigens in children with asymptomatic *P. falciparum* infections cleared by DP.

#### 2.3. Microscopy or examination of blood smears

Thick and thin blood smears were stained with 10% Giemsa after fixing thin smears with methanol. Asexual parasite densities were determined by counting against 200 leukocytes, and converting to parasites per μL by assuming a standard leukocyte count of 8000/μL (Greenwood and Armstrong, 1991). At least 100 high power fields were examined before a thick smear was declared negative. Each slide was read independently by two experienced readers, and the geometric mean reading of the two replicates used for analysis. A third reader read the slide in cases where the parasite density estimates of the first two readers differed by more than 10%. Sexual parasite rate and density were determined by counting against 500 leukocytes and converted to parasites per μL as for asexual parasites (Drakeley et al., 2004).

#### 2.4. Species identification by nested PCR

DNA was extracted from filter paper blood spots from visits 1 and 5 respectively in a 96-well plate using Chelex resin as previously described (Oguku et al., 2011). *Plasmodium* species were identified by nested PCR amplification of the small sub-unit ribosomal genes as previously described (Snounou et al., 1993) with the following second round PCR primers and amplicon sizes:
**P. falciparum primers:** rfal1 + rfal2 205 bp product

**P. malariae primers:** rmal1 + rmal2 144 bp product

**P. vivax primers:** rviv1 + rviv2 120 bp product

**P. ovale primers:** PovaFWD + RVScommon 375 bp product

Newly designed *P. ovale* spp. primers were deployed because the original pair of primers described by Snounou et al. (1993) do not amplify *P. o. wallikeri*.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PovaFWD</td>
<td>5'-CTGTTCTTTGCATTCCTTATGC-3'</td>
</tr>
<tr>
<td>RVS common</td>
<td>5'-GTATCTGATCGTCTTCACTCCC-3'</td>
</tr>
</tbody>
</table>

For the second round amplification, 1 µl of first round PCR product was amplified in a 20 µl reaction containing 10 µl of HotStar Taq master mix (Qiagen, UK) and 0.2 µM of each primer. PCR products were resolved on 2% agarose gels.

2.5. Discrimination between *P. o. curtisi* and *P. o. wallikeri*

*P. o. curtisi* and *P. o. wallikeri* were distinguished from each other by nested PCR amplification of the gene coding for tryptophan-rich antigen, and fractionation on agarose gels, or by direct sequencing of pog3p and porbp2 amplicons as previously described (Oguike et al., 2011).

2.6. Statistical analysis

Associations between binary variables were tested using the chi-squared statistic, with Fisher’s exact correction for comparisons in which any expected value was less than 6. Estimation of summary statistics and all tests of association were performed in STATA software (Stata 12.0, Statacorp, Texas, US).

3. Results

3.1. Parasite carriage by microscopy

Single-read microscopy of all 274 children immediately following screening identified 165 with positive blood films (geometric mean density 1.87 parasites µL⁻¹ peripheral blood, range 0–428 µL⁻¹). Consent for inclusion in the longitudinal study was obtained from parents/guardians for 155 of these children. Definitive double-read microscopy carried out after completion of the study found an additional 16 children positive for *P. falciparum* trophozoites that had not been enrolled in the longitudinal study. Twenty-one of the enrolled children (13.6%) carried patent gametocytes of *P. falciparum*, 14 of whom carried concurrent asexual parasites. Further, the microscopists reported that the blood films of a small number of children with visible *P. falciparum* trophozoites also harboured blood-stage parasites of either *P. malariae* (24 children) or *P. ovale* spp. (3 children) (Fig. 1A).

At visit 2 (7 days after the first visit), DP treatment was given under observation to all 155 enrolled participants and a further blood film and filter paper collected. Double-read microscopy found that 55 of the 155 enrolled children no longer harboured patent asexual parasites, and that two of seven children who at visit 1 had circulating gametocytes only now harboured detectable asexual parasites of *P. falciparum*. These data show that a number of children in the study harboured fluctuating parasite densities very close to the limit of detection of microscopy, and hence parasite detection was often discordant between the two visits, despite
Table 1

Mixed parasite species carriage detected by PCR at enrolment and 3 weeks post-treatment Data on parasite carriage is presented as a binary code for each of the three species (with all ovale infections considered together), where “0” denotes the absence of a species, and “1” denotes its presence by nested PCR. Only children with PCR data indicating the presence of at least one species are shown at each timepoint.

<table>
<thead>
<tr>
<th>Pf/Pn/Po PCR code</th>
<th>Visit 1 (N = 270)</th>
<th>Visit 5: 3 weeks post-treatment (N = 152)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Freq.</td>
<td>Percent</td>
</tr>
<tr>
<td>000</td>
<td>55</td>
<td>20.4</td>
</tr>
<tr>
<td>001</td>
<td>4</td>
<td>1.5</td>
</tr>
<tr>
<td>010</td>
<td>3</td>
<td>1.1</td>
</tr>
<tr>
<td>011</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>100</td>
<td>115</td>
<td>42.6</td>
</tr>
<tr>
<td>101</td>
<td>20</td>
<td>7.4</td>
</tr>
<tr>
<td>110</td>
<td>53</td>
<td>19.6</td>
</tr>
<tr>
<td>111</td>
<td>19</td>
<td>7.0</td>
</tr>
<tr>
<td>Total</td>
<td>270</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Pf: Plasmodium falciparum; Pn: P. malariae; Po: P. ovale spp.

3.2. Species-specific PCR to detect parasite carriage prior to treatment

As expected, retrospective PCR analysis of DNA extracted from all 274 blood spots collected at visit 1 found 45 additional sub-patient P. falciparum infections. It was also shown that a high proportion of enrolled individuals were found to carry non-falciparum parasite species. A further 15 children previously parasite-negative by microscopy harboured P. malariae, and 8 were PCR positive for P. ovale spp., many children harbouring multi-species infections (Table 1). In total, 215 individuals out of 270 evaluable PCR tests (79.6%) were parasite positive for at least one species. Whereas 115 children among the 215 who were parasite positive were found to harbour P. falciparum alone, eight individuals were infected only with non-falciparum species, and 92 with P. falciparum and at least one of the other species. These findings are summarised using a simple binary species code in Table 1. The overall prevalence of P. malariae in all children tested was 28% (76 children), and that of P. ovale spp. was 16.3% (44 children). Taking the median age of 10 years as a cut-off, evidence was found that PCR-detected carriage was more common in the older group for both P. falciparum (OR: 2.21; 95% CI: 1.18–4.25; P = 0.0082) and P. malariae (OR: 2.07; 95% CI: 1.17–3.69; P = 0.0077), but not for P. ovale spp. (OR: 0.860; 95% CI: 0.422–1.73; P = 0.651). Further, the occurrence of either or both non-falciparum species at visit 1 was much more likely in children who were also positive for P. falciparum by PCR (odds ratio OR: 5.50; 95% CI: 2.43–14.0; P < 0.001). This relationship with P. falciparum carriage was also seen for P. malariae or P. ovale spp. when considered separately (OR: 7.87; 95% CI: 2.73–30.8; P < 0.001 and OR: 2.69; 95% CI: 0.990–9.14; P = 0.400, respectively). Thus 67 of the 155 children treated with DP in this study and followed up also harboured PCR-confirmed non-falciparum malaria infections, which were thus inadvertently exposed to one of the currently recommended ACT regimens.

P. o. curtisi and P. o. wallikeri are indistinguishable by microscopy, and so PCR amplification of the potra and pog3p genes was performed to discriminate which species were present in the 44 children identified by nested PCR as carrying P. ovale spp. (Oguke et al., 2011). Electrophoretic fractionation of potra amplification products, or direct sequencing of pog3p amplicons was able to identify P. o. curtisi in 27 individuals and P. o. wallikeri in 7 individuals. (In the remaining 10 children there was insufficient material to make a species-level identification.) Two children appeared to harbour both species by the potra assay. As individuals with both species present simultaneously have only recently been described (Fancony et al., 2012; Fuehrer et al., 2012), we investigated these two children in more detail, repeating the original tests, and using other discriminatory loci (Sutherland et al., 2010). The presence of both P. o. curtisi and P. o. wallikeri sequences at each of the loci potra, pog3p, and porbp2 was confirmed in both individuals, as sequences identical to those described for both species in previous studies were found (Sutherland et al., 2010; Oguke et al., 2011). Thus we conclude that each of these children harboured both parasite species that cause ovale malaria, supporting the recent demonstration that these related species are able to infect a single host simultaneously.

3.3. Parasite carriage following DHA-piperazine

Of 155 children microscopy positive on visit 1, treated with DP on visit 2 and followed up, four remained positive for P. falciparum trophozoites by microscopy at visit 5 (Fig. 1A). Species-specific PCR gave four different species codes for these individuals (Table 1): 100, 110, 111 and 001. Thus, in one case, the microscopists identified P. ovale spp. parasites as a recurrent P. falciparum infection. Nested PCR testing of all samples at visit 5 identified 22 parasite positive individuals, a prevalence of recurrent parasitaemia 3 weeks after treatment of 14.5%. Unexpectedly, in only 16 of these individuals was P. falciparum the sole parasite species detected by nested PCR (Table 1; Fig. 1B). Thus post-treatment parasitaemia with non-falciparum malaria was detected in 6 individuals, and of these four were positive for this same species prior to treatment, two children each for P. ovale and P. malariae. The proportion of post-treatment infections positive for DNA from P. ovale spp. was 18%, which is similar to the baseline proportion of 20.5%. Two of these were confirmed as P. o. curtisi by genotyping at both potra and pog3p loci, whereas insufficient material was available to definitively identify the remaining two P. ovale spp. infections. DNA from P. malariae was detected in 13.6% of post-treatment infections, compared to 35.4% of infections prior to treatment.

4. Discussion

This study examines, in asymptomatic Ghanaian school children, the effect of ACT against the three less-common parasite species which infect people in sub-Saharan Africa: P. malariae, P. o. curtisi and P. o. wallikeri. These species are generally considered sensitive to standard antimalarial drugs including chloroquine (Collins and Jeffery, 2007; Mueller et al., 2007), yet we have shown using PCR-based detection that a small number of these infections recurred 3 weeks after treatment of asymptomatic parasite-positive individuals. As DP is known to provide good post-treatment prophylaxis (Four ABC Study Group, 2011), this finding was unexpected. In addition, we provide confirmation, using a multi-gene typing approach, that P. o. curtisi and P. o. wallikeri can be found simultaneously in a single individual, as two of our school children were found to harbour both ovale parasite types prior to treatment. Taking these results together with recent findings from Bangladesh and Angola (Fancony et al., 2012; Fuehrer et al., 2012), the weight of evidence is now firmly against our previously stated hypothesis that human blood groups may restrict (whether malaria or not) in the older children and thus less frequent use of antimalarial drugs. If this suggestion is true, the absence of such an age relationship for P. ovale spp. can be
attributed to the ability of *P. o. curtisi* and *P. o. wallikeri* to relapse from dormant liver-stage hypnozoites once antimalarial drugs have cleared from the host bloodstream (Nolder, Oguike & Sutherland, unpublished data). Further, *P. malariae* and *P. ovale* spp. were significantly more commonly detected by PCR in individuals that were also PCR-positive for *P. falciparum*. Whereas hitherto unknown biological mechanisms may be responsible for this phenomenon, a simple and parsimonious explanation is that all three parasites are circulating in the same *Anopheles* mosquito populations, and thus children more exposed to *P. falciparum* infection because they live near mosquito habitat, have an unscreened house or do not use a bednet will also be more often exposed to the other *Plasmodium* species. Identification of *Plasmodium* species in blood-fed anophelines from this area would help to test this explanation. Further detailed studies of the associations between age, multi-species parasite carriage, quality of housing, exposure to biting mosquitoes and patterns of community drug use in African endemic areas are clearly needed.

The presence of PCR-detectable parasitaemia 3 weeks after ACT treatment in some of the study children is of concern. However, *in vivo* antimalarial drug efficacy cannot be meaningfully estimated from treatment outcomes in asymptomatic infections, and PCR positivity is not (yet) validated as a primary endpoint for clinical trials of antimalarial therapy. Bearing in mind these caveats, we nevertheless expected a higher rate of complete parasite clearance among the asymptomatic cases of *Plasmodium* spp. infection in our study. As piperaquine is considered to have a prophylactic effect lasting more than 3 weeks (d’Alessandro, 2009; Four ABC Study Group, 2011), emergent blood-stage infections from recent mosquito bites should also have been prevented. The observation that DNA from non-falciparum parasite species can be detected within 3 weeks of treatment with an effective ACT in school children was particularly unexpected, suggesting continuing asexual parasitaemia of *P. malariae* and *P. ovale* spp. in these individuals. There are a few possible alternative hypotheses to explain our findings of persistent sub-patent parasitaemia. Firstly, DNA from dead malaria parasites may persist post-treatment, either in circulating soluble form or in phagocytes that have engulfed parasites, and deliver a “false-positive” signal in diagnostic PCR tests (Sutherland and Hallett, 2009). However, there is no direct evidence to support this, and persistent DNA from dead parasites is unlikely to remain detectable after 3 weeks, particularly with the low starting parasite densities in our study subjects. A second plausible explanation is that the DNA we have detected is only from the gametocytes (sexual stages) of these species. It is known for *P. falciparum* that gametocyte carriage after DP treatment is more common than for other regimens such as artemether-lumefantrine (Four ABC Study Group, 2011), and that gametocytes of this species remain circulating in a proportion of ACT-treated malaria patients long after apparent clearance of asexual stages (Targert et al., 2001). Gametocytes of *P. malariae* and *P. ovale* spp. circulate in the peripheral blood of infected people at the same time as asexual stage parasites, and it is generally assumed that they have only a short lifespan *in vivo*. However, knowledge of the patterns of gametocyte carriage for these species is mostly drawn from malaria in travelers, experimental infections in naive volunteers and from studies of therapeutic malaria induced in syphilis patients in the mid 20th century (Garnham, 1966). This knowledge is therefore unlikely to accurately reflect the actual patterns in endemic country populations. A full understanding of the effect of treatment on the gametocytes of *P. malariae* and *P. ovale* spp. can only be gained once new methods of detection for these sexual stages are developed.

Given that the arguments above may not explain our results, the apparent “failure” of ACT to clear *P. malariae* and *P. ovale* spp. in Ghanaian school children requires further consideration. *P. ovale* spp. are known to relapse from dormant liver-stages analogous to the hypnozoites of *P. vivax* and *Plasmodium cynomolgi* (Garnham, 1948; Shortt and Garnham, 1948) and both ovale species are known to cause relapse clinical malaria months or years after exposure to infected mosquitoes (Sutherland et al., 2010). There is thus reason to think that *P. ovale* spp. relapses may follow treatment of *P. falciparum* infections, as is described for *P. vivax* in Asia (Douglas et al., 2011). However, given the long half-life of piperaquine (d’Alessandro, 2009; Four ABC Study Group, 2011) and the relatively short period of follow-up in the present study (3 weeks) a relapse emergence from the liver would have been expected to encounter reasonable plasma levels of piperaquine and thus fail to reach detectable levels (even by PCR) in this short time. Thus there may be some other intrinsic survival mechanism that *P. ovale* spp. can deploy to evade drug clearance. *P. malariae* on the other hand is thought to lack the ability to relapse from dormant liver stages (Shortt and Garnham, 1948; Ciucă et al., 1964; Collins and Jeffery, 2007). The 72 h (quartan) intra-erythrocytic cycle time may make this species particularly well suited to evading the effect of most artemisinin regimens, as each dose generates only a short pulse of active plasma DHA which is very rapidly cleared. *P. malariae* infections may therefore be more likely to leave a few survivors after each dose of artemisinin is administered. There are concerns that the current amount of DHA used in the fixed combination DP as given to children results in under-dosing (d’Alessandro, 2005; Tarning et al., 2012), and longer dosage regimens of ACT may be required for complete clearance of *P. malariae* infections. In addition, this parasite is well known to possess a capacity for dormancy, lasting decades in some well documented cases (Vinetz et al., 1998). Although the mechanism or host tissue reservoirs are unknown, it is plausible that this same ability may assist in evasion of antimalarial therapy in this species.

Equally of concern is the persistent detection of *P. falciparum* at 3 weeks in approximately 15% of DP-treated individuals in this study. By microscopy, 4 individuals were recorded as positive for *P. falciparum* asexual parasites 3 weeks after treatment, whereas PCR identified 10 individuals positive for this species, some of which may be carrying only gametocytes. *A priori*, it could be assumed that asymptomatic infections with *P. falciparum*, at low parasite densities, would be easier to clear with efficacious antimalarial regimens. On the contrary, we find a number of PCR-detectable infections only 3 weeks after DP. A possible explanation for this is that in chronic, asymptomatic infections malaria parasites are in a steady-state relationship with the host, and do not elicit immune responses that are capable of participating in drug-induced parasite clearance; thus drug efficacy may be partially compromised against such infections. In contrast, symptomatic infections are by definition immunogenic, and thus the outcome of treatment is the sum effect of drug and both innate and acquired immune responses (Djimdé et al., 2003; Diallo et al., 2007). A potential mechanism for such a phenomenon is the expression, by parasites in a chronic infection, of surface antigen PIEMP1 variants that have weak adhesive properties and are poorly immunogenic (Jensen et al., 2004; Bull et al., 2005). This would explain a relative lack of immune clearance in comparison to symptomatic infections, but does not explain how these parasites evade drug-induced killing.

5. Conclusions

This report raises two areas of concern for programmes seeking to eliminate malaria in sub-Saharan Africa. Firstly, co-infecting non-falciparum species were found by PCR to be common in asymptomatic individuals with patent *P. falciparum* parasitaemia, and the deployment of an ACT thought efficacious against all three
species failed to completely clear *P. malariae* and *P. ovale* spp. in approximately 10% of individuals over 3 weeks of follow-up. The reason for persistent detection of parasites is unknown. Secondly, although ACT clearance of asymptomatic *P. falciparum* infections was efficacious using a microscopy endpoint, PCR-detectable parasites were identified within 21 days of treatment. The role of the immune system in assisting drug-induced parasite clearance may be compromised in chronic low-grade infections of this parasite.

**Acknowledgements**

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