RESEARCH ARTICLE

Comparing drug regimens for clearance of malaria parasites in asymptomatic adults using PCR in Kilifi County, Kenya: an open-label randomised controlled clinical trial (MalPaC) [version 1; peer review: awaiting peer review]

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Abstract

Background: To restrict trial endpoints to infections acquired after vaccination in Phase Ib trials of candidate malaria vaccines, participants are treated with anti-malarial drugs to clear existing infections. Anti-malarial drugs with a long half-life may inhibit the acquisition of new infections. This study evaluated the effects of three anti-malarial drug regimens on the clearance of existing infections and acquisition of new infections.

Methods: An open-label randomised controlled trial (MalPaC) was conducted between November 2013 and February 2014. Ninety adults were randomised 1:1:1 to receive one of three treatments: atovaquone/proguanil and artesunate (AP+AS); artesunate (AS); or sulphadoxine-pyrimethamine, artesunate, and primaquine (SP+AS+PQ). Parasite monitoring was determined over 84-day follow-up by assessing Plasmodium falciparum positivity by 18s qPCR, live and sexual stage parasites by RT-PCR, and recrudescence of infections by msp2 genotyping.

Results: At enrolment, parasite prevalence by qPCR was 44% (40/90, day 0), which fell to 10% (9/90, day 16), then rose to almost the initial rates by day 84 (39%, 35/90). Individuals treated with AS and SP+AS+PQ were more likely to have higher qPCR positive rates compared to participants treated with AP+AS in the immediate post-treatment phase (days 16-28)
(OR=7.7 [95%CI 4.6-12.8] p<0.0005 and OR=4.2 [95%CI 2.6-6.8] p<0.0005, respectively). In the immediate post-treatment phase, qPCR positivity was less likely associated with evidence of live parasites and gametocytaemia. Prevalence of “old”, “new” or “undetectable” infections did not differ significantly over time or drug regimen. However, participants on the AP+AS drug regimen were less likely to have parasite infection recrudescence compared to participants treated with AS and SP+AS+PQ.

**Conclusion:** Falciparum DNA remained detectable by PCR post-treatment with incomplete parasite clearance regardless of drug regimen. Though AP+AS drug regimen may also have partially suppressed the acquisition of new infections during post-treatment follow-up.

Trial registration: Pan African Clinical Trials Registry, 22nd of August 2013, PACTR201309000625311.

**Keywords**
Malaria, Parasite clearance, PCR monitoring, Atovaquone/proguanil, Artesunate, Primaquine, Sulphadoxine-pyrimethamine, antimalarials

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Introduction

*Plasmodium falciparum* malaria is an important cause of morbidity and mortality in endemic populations in Africa. Interventions including insecticide treated mosquito nets (ITN) and anti-malarial drugs have reduced mortality globally; however, more interventions are required for elimination and/or eradication. A vaccine is urgently needed, and several candidates are undergoing testing. Prior to large scale field trials, Phase II studies are undertaken to obtain preliminary evidence of efficacy. These can include controlled human malaria infections or randomized field trials including adults or children. Adults in endemic regions have immunity to clinical malaria, and hence trials involving adults normally use the acquisition of infection as an endpoint. In these trials, anti-malarial drugs are used prior to surveillance for malaria infection to differentiate between new infections and sustained infections acquired before vaccination, particularly for pre-erythrocytic vaccines which can only act against new infections acquired after vaccination and not old infection acquired prior to vaccination. The ideal anti-malarial drug(s) for this purpose should be able to clear parasites but not inhibit the acquisition of new infections during follow-up as this would undermine the ability to detect vaccine efficacy.

Clearance of parasite post-treatment can be assessed using PCR, microscopy, or rapid diagnostic tests (RDTs). PCR is more sensitive at detecting malaria parasites and increases the power of studies to detect endpoints. PCR can detect parasites at low density, detecting parasites as low as 2 to 5 parasites/μl of blood depending on the volume of blood used for PCR extraction, whereas microscopy and RDTs can detect parasite densities of above 50 parasites/μl. Furthermore, PCR based genotyping of *P. falciparum* parasites is also used to differentiate new from recrudescence infections.

Different antimalarial drugs have been used for clearing parasitaemia prior to surveillance in previous Phase IIb trials for malaria vaccines. Sulphadoxine-pyrimethamine (SP) was given to participants to clear blood stage *P. falciparum* infections two weeks before administering the third dose of the RTS,S/AS02 vaccine in a study in The Gambia to test the efficacy of RTS,S/AS02. The acquisition of infection(s) was then monitored by blood film microscopy. In another study in The Gambia, participants, who were randomized to receive either DNA ME-TRAP followed by MVA ME-TRAP or rabies vaccine (control group), were given a dose of SP two weeks before the last vaccination, with follow-up by microscopy. Furthermore, another trial using PCR monitoring for malaria parasites rather than microscopy, parasite clearance was achieved using Lap/Dap (a combination of chlorproguanil and dapsone), artesunate and primaquine were used. Lap/Dap has since been withdrawn from public use because of its strong association with haemolytic anaemia. Further studies in Kenya with PCR monitoring have used atovaquone/proguanil with artesunate. However, there are no studies comparing the different antimalarial drugs in clearing parasites in the context of vaccine trials and their impact on the acquisition of new infections post-vaccination. In this study, we compared the clearance of PCR-detectable malaria parasites in adult participants in a Phase IIb clinical trial following the administration of three different drug regimens.

Methods

Trial registration

The trial was registered on the Pan African Clinical Trials Registry on the 22nd of August 2013. Trial identifier number: PACTR201309000625311.

Ethical statement

The study was approved by the Kenya Medical Research Institute Scientific and Ethics Review Unit (KEMRI-SERU, SSC 2565) and the University of Oxford Tropical Ethics Research Committee (OxTREC, 1034–13). The trial was also approved by the Kenyan regulatory authority, Pharmacy and Poisons Board (ECT/13/08/02). Informed consent was sought at the study dispensaries by study clinicians with the assistance of experienced field workers. Potential volunteers received verbal explanations and study information sheets that were translated in relevant local languages and discussed any concerns that required clarifications with investigators. The volunteers were asked to provide written informed consent before being recruited to participate in the study. Volunteers who were unable to read and write were asked to thumb print the consent form if they wished to consent, and this was counter-signed by an independent witness. Personal information was handled with utmost confidentiality and documents secured in the Clinical Trials Facility. Personal information was not entered into the electronic database.

Study site

This study was carried out in Junju, Kilifi County in Kenya, an area of moderate to high transmission with estimates of 22 infective bites/person/year between November 2013 to February 2014. ITN use in the study area increased from 55.9% in 2009 to a peak of 82.6% in 2013 partly due to a mass distribution of ITNs in the same year.

Inclusion and exclusion criteria

Local administrative and community leaders were first sensitized about the project. Public meetings were then arranged in the study villages to inform the communities of the study. These meetings were facilitated by the investigators. Field workers helped to identify potential volunteers where they conducted one-on-one meetings with them to ensure understanding of the information sheet and the consent form. Individuals who then felt the trial was appropriate for them to participate in were invited to attend a formal screening visit in the study dispensary where the study clinicians sought their informed consent with the help of experienced field workers. Residents of the study area aged between 18 and 50 years, who consented to participate in the study, were included in the study. Upon consenting, volunteers were screened for any clinically significant acute and chronic illnesses by physical examination and laboratory tests. Volunteers were excluded if they had: (1) significant medical conditions or low levels of haemoglobin (less than 11.3 g/dl for men and less than 10 g/dl for women); (2) blood transfusion within a month preceding enrolment; and (3) enrolled in
another clinical trial under 12 weeks preceding enrolment in this study. In addition, female volunteers were screened for pregnancy on recruitment and on the first day of drug administration. Pregnant and lactating women and those unwilling to use contraception for the duration of drug treatment were excluded from the study. Recruitment and screening of volunteers was continued until 90 eligible volunteers were identified.

**Sample size considerations**
In a previous study, 60% of adults were PCR positive prior to drug treatment. At 7 days after treatment 30% were positive and only 5% subsequently had high-level positive PCR results.

According to the primary objective, we needed to detect the difference between 30% positivity at 7 days and lower levels, which would ideally be 1 to 2%. Using STATA software version 12 for a comparison of proportions at p=0.05 and power=0.8, this suggests a sample size of 30 subjects per group.

To detect the difference between 5% late high-level positivity and our estimated proportion in the absence of any long-term drug effect, at 40%. Using STATA software version 12 for a comparison of proportions at p=0.05 and power=0.8, this suggests a sample size of 20 subjects per group.

Since the former calculation required a larger sample size, a sample size of 30 per study group was used.

**Study design**
This was an open-label, single site, parallel, randomised controlled trial involving adults.

**Randomisation.** Ninety healthy asymptomatic adult participants were recruited to participate in the study and randomized by an independent statistician to three different groups in the ratio 1:1:1 to receive one of the following drug regimens: Group 1: atovaquone/proguanil (4 tablets each of 250mg/100mg taken once daily for 3 days) and artesunate (200mg once daily for 3 days) (AP+AS); Group 2: artesunate (400mg on day 1 and then 200mg once daily for 6 days) (AS); or Group 3: sulphadoxine-pyrimethamine (3 tablets 500mg/25mg taken once on day 1), artesunate (200mg once daily for 3 days) (AS), and primaquine (1 dose of 15mg taken once on day 1) (SP+AS+PQ).

Randomization was carried out after screening had been done and participant eligibility has been confirmed. A two stage randomization process was carried out. 20 G6PD deficient participants were first randomised to Group 1 and 2 by simple randomization. The remaining 70 (without G6PD deficiency) were randomly assigned in a ratio of 2 to 3 to group (1 and 2) and group 3 respectively by simple randomisation. This was to avoid the potential for haemolytic anaemia if a G6PD deficient volunteer is given primaquine (Group 3).

The random allocation sequence was generated by an independent statistician using STATA version 13, and this allocation was indicated on a card that was sealed inside an opaque envelope with the study ID number of eligible participants written on it. Inside the envelopes were cards printed with the study ID corresponding to what had been written on the envelope and treatment allocation stuck on them. Participants were enrolled by the study clinicians after they did a final eligibility check, after which they handed over the patient case report form (CRF; **Extended data***) and sealed randomized treatment (with a study number corresponding to the study ID number on the CRF) to the study nurse to administer the randomised treatment. Drug administration commenced on day 0. The laboratory scientists were blinded to the drug allocation until the end of the study, but the clinical investigators, participants and field teams were not blinded.

**Malaria parasite monitoring.** Malaria parasite monitoring was done by PCR for markers for asexual and sexual parasites. This was done at screening for eligibility followed by a total of 25 time points (2,249 samples) after enrolment into the study. In the first 3 weeks after enrolment, venous blood samples in ethylenediaminetetraacetic acid (EDTA) were taken and tested by PCR three times per week, subsequently, samples were taken for PCR twice per week for 3 weeks and then once every week for another 6 weeks. For qPCR analysis, 0.5ml of whole blood was collected. For RT-qPCR parasite monitoring, 100 µL of whole blood samples and the appropriate amount of trizol was added and the sample kept at −80 °C until analysed.

**qPCR for detection P. falciparum parasites.** DNA was extracted from 500 µL of whole blood after white cell depletion using a previously described method of filtering4. The DNA was eluted in 50 µL, from which 15 µL of DNA (5 µL run in triplicate) were amplified by quantitative PCR using a TaqMan assay for *P. falciparum* multicopy 18S ribosomal RNA gene21. Quantification was on an Applied Biosystems 7500 Real-Time PCR System with quantification by Applied Biosystems 7500 software v2.0.6. Samples were analyzed in singlet wells. Three negative control wells (non-template control) and 7 serial dilutions of DNA extracted from in vitro parasite cultures were included as standards on each plate in triplicate as described previously8. Plates failing quality control standards were repeated. This was done for all the 2,249 samples across all 25 time points.

**RT-qPCR for live parasites and sexual parasite markers.** A subset of samples were analysed for asexual and sexual parasites by RT-qPCR. To understand the reasons for persistent DNA positivity at low parasite densities, the time points with low qPCR parasitaemia (<100 parasites/µL) were particularly selected. Baseline samples (day 0 samples) for comparison were needed, and thus included for all respective participants meeting the criteria of low parasitaemia. In addition, the respective time points with the highest qPCR parasitaemia for the respective participants were included in the analysis with two other randomly selected time points of qPCR positivity per participant.

RNeasy mini extraction kit (QIAGEN) was used to extract total RNA. The quantity and purity of the RNA was assessed by a Nanodrop spectrophotometer (Thermo Scientific). RNA was treated with DNase using the Turbo DNA-free kit (Invitrogen) and 2 µg used for complementary DNA (cDNA) synthesis.
by SuperScript VILO cDNA Synthesis Kit (Invitrogen). For *P. falciparum*, multiplex 18S ribosomal RNA gene or Pf25 gametocyte detection\(^1\), total reaction volume of 20µl: 10µl of 2X SYBR\(^®\) Green Master mix (Applied Biosystems\(^®\)), 5µl of primer (either target or housekeeping) and 5µl of cDNA sample was used to amplify target sequences by reverse transcriptase PCR. All RT-PCR reactions were run on an ABI 7500 Real-Time PCR system (Applied Biosystems\(^®\)). Target primer sequences were assayed alongside housekeeping gene primers\(^2\). Positive and negative control samples were included in all RT-PCR reactions. For positive control, asexual parasites of 3D7 strain were either cultured up to gametocyte stages as described elsewhere or up to asexual stages\(^2\). RNA was extracted from day 14 mature gametocytes and cDNA synthesized using the above-mentioned kits and included in the gametocyte detection assays. Uninfected red blood cells were also taken through the same process and used as a negative control. Non-template control was also included as a control.

**Msp2 genotyping.** Msp2 genotyping was performed to differentiate between old (i.e. recrudescent from pre-treatment samples) and new infections for *P. falciparum* infections within individuals over time for time points with qPCR parasite positivity. The qPCR positive DNA samples were analyzed by a previously described nested PCR method in which fluorescently labeled primers specific to the two allelic types (FC27 and IC/3D7) were used in the nested reaction, followed by fragment sizing by multi-coloured capillary electrophoresis in a DNA sequencer\(^3\).

**Endpoints.** The primary endpoint was to compare drug regimens for the first episode of *P. falciparum*, defined as two or more consecutive blood samples confirmed positive by PCR for *P. falciparum*. The secondary endpoints were: 1) To determine the percentage of PCR results that are confirmed by RNA positive results indicating live *P. falciparum* parasites; 2) To compare the percentage of positive results after day 7 after drug treatment to examine clearance rates and longitudinal patterns of PCR positivity to distinguish re-infection from failure to clear parasites.

**Statistical analysis**

STATA software version 15 was used to perform statistical analysis\(^8\). Clearance rates following the three drug regimens were compared to determine the optimal drug regimen. These were expressed as counts and as percentages of samples that were PCR positive at various time points following treatment. The Lowess curve was used to show the trend in malaria infections. Concordance between various malaria parasite monitoring strategies was assessed using the chi-square test. The relationship between demographic characteristics of study participants and PCR positivity was assessed using Logistic regression. A multilevel logistic regression model was used to adjust for the non-independence of repeated measures per individual over time.

Classification of infections was done using *msp2* genotyping. Two genotypes were considered different if they were of different allelic types (FC27 or IC/3D7) or if their fragment length differed by more than 3 base pairs within the same allelic type. An infection was classified as “old” if its genotype was first detected before visit day 7 or “new” if its genotype was not seen before visit day 7. A genotype was only considered once per individual for classification as “new” or “old” on first identification after day 7 (e.g. a genotype detected on days 13, 15 and 17 was only considered on day 13). An infection was classified as undetected if the PCR sample was positive, but the genotyping failed. The Kruskal-Wallis equality of populations test was used to compare differences in prevalence of the type of infection.

**Results**

**Demographic characteristics**

In total 167 participants were recruited and screened for eligibility and enrolment into the study. 90 participants met the eligibility criteria, were enrolled and randomised into one of the drug regimen groups in the study (Figure 1). Treatment groups were balanced for gender, age and bed net use (Table 1).

**Parasite prevalence following treatment**

There was 99.9% completion rate with 89 participants completing all 25 study visits and only one participant not attending the last visit on day 84. A total of 2,249 samples for the 25 timepoints were collected and analysed for the presence or absence of malaria parasites by qPCR.

In total, 44.4% (40/90) participants were positive for *P. falciparum* specific 18s qPCR on enrolment (day 0) and the positivity rate fell to 24.4% (24/90) participants and 16.7% (15/90) participants by day 9 and day 14, respectively. Rates then fell further by day 16 to 10.0% (9/90) participants. The prevalence rose to 16.7% (15/90) participants by day 18 and 39.3% (35/90) at the end of monitoring (day 84) (Table 2). When aggregated by time period, there was an overall reduction in the qPCR positive rate for malaria parasites from 32.7% (147 out of 450 samples) during the drug treatment period (between day 0 and 7) to 20.4% (55/270) in the immediate post-treatment period (i.e. between day 9 and day 14) and to 14.1% (76/540) during post-treatment follow-up (between visit days 16 and 28) (Figure 2).

During post-treatment follow-up (between days 16 and 28), the highest positivity rate was in those who received AS at 22.8% (41/180), while the lowest was in those who received AP+ AS drug regimen at the rate of 7.2% (13/180). The trend in malaria infections by drug regimen was evident as shown by the Lowess curve (Figure 3).

**Predictors of qPCR positivity.** To assess the determinants of parasite positivity by qPCR including day, drug treatment, age, and bed net use in the reference category for drug treatment, a multivariable logistic model was used. Compared with AP+AS, participants treated with AS were more likely to be parasite qPCR positive during post-treatment follow-up (between day 16 and 28, OR=7.7 (95%CI 4.6-12.8) p<0.0005) and in the subsequent follow-up period (between day 31 and 56,
Table 1. Demographic characteristics of the study participants.

<table>
<thead>
<tr>
<th></th>
<th>AP+AS</th>
<th>AS</th>
<th>SP+AS+PQ</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male, N (%)</td>
<td>14 (46.7)</td>
<td>13 (43.3)</td>
<td>19 (63.3)</td>
<td>46 (51.1)</td>
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<tr>
<td>Female, N (%)</td>
<td>16 (53.3)</td>
<td>17 (56.7)</td>
<td>11 (36.7)</td>
<td>44 (48.9)</td>
</tr>
<tr>
<td>Age (years) Mean (SEM)</td>
<td>30.5 (1.51)</td>
<td>30.9 (1.53)</td>
<td>30.7 (1.52)</td>
<td>30.7 (0.87)</td>
</tr>
<tr>
<td>Bed net use, N (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No bed net use</td>
<td>10 (33.3)</td>
<td>4 (13.3)</td>
<td>9 (30.0)</td>
<td>23 (25.6)</td>
</tr>
<tr>
<td>Treated bed net with no holes</td>
<td>8 (26.7)</td>
<td>16 (53.3)</td>
<td>10 (33.3)</td>
<td>34 (37.8)</td>
</tr>
<tr>
<td>Treated bed net with holes</td>
<td>10 (33.3)</td>
<td>6 (20.0)</td>
<td>9 (30.0)</td>
<td>25 (27.8)</td>
</tr>
<tr>
<td>Untreated bed net</td>
<td>2 (6.7)</td>
<td>4 (13.3)</td>
<td>2 (6.7)</td>
<td>8 (8.9)</td>
</tr>
<tr>
<td>Total</td>
<td>30 (100.0)</td>
<td>30 (100.0)</td>
<td>30 (100.0)</td>
<td>90 (100.0)</td>
</tr>
</tbody>
</table>

OR=2.1 (95%CI 1.2-3.5) \( p=0.011 \). Similarly, those who were treated with SP+AS+PQ were more likely to be parasite qPCR positive over the same time periods respectively: between visit days 16 and 28 (OR=4.2 (95%CI 2.6-6.8) \( p<0.0005 \)) and between days 31 and 56 (OR=2.8 (95%CI 2.0-4.1) \( p<0.0005 \)).

Bed net use was significantly associated with lower likelihood of qPCR positivity (Figure 4).

The impact of clustering by visit day and volunteer was examined using a) a two-level hierarchical model and b) using...
Table 2. The prevalence of positive qPCR participants by drug regimen and visit days.

<table>
<thead>
<tr>
<th>Visit day</th>
<th>AP+AS (n=30)</th>
<th>AS (n=30)</th>
<th>SP+AS+PQ (n=30)</th>
<th>Overall (n=90)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>17 (56.7%)</td>
<td>12 (40.0%)</td>
<td>11 (36.7%)</td>
<td>40 (44.4%)</td>
</tr>
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<td>1</td>
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<td>10 (33.3%)</td>
<td>10 (33.3%)</td>
<td>34 (37.9%)</td>
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<tr>
<td>2</td>
<td>14 (46.7%)</td>
<td>9 (30.0%)</td>
<td>7 (23.3%)</td>
<td>30 (33.3%)</td>
</tr>
<tr>
<td>4</td>
<td>11 (36.7%)</td>
<td>6 (20.0%)</td>
<td>5 (16.7%)</td>
<td>22 (24.4%)</td>
</tr>
<tr>
<td>7</td>
<td>10 (33.3%)</td>
<td>6 (20.5%)</td>
<td>5 (16.7%)</td>
<td>21 (23.3%)</td>
</tr>
<tr>
<td>9</td>
<td>10 (33.3%)</td>
<td>8 (26.7%)</td>
<td>4 (13.3%)</td>
<td>22 (24.4%)</td>
</tr>
<tr>
<td>11</td>
<td>6 (20.0%)</td>
<td>4 (13.3%)</td>
<td>8 (26.7%)</td>
<td>18 (20.0%)</td>
</tr>
<tr>
<td>14</td>
<td>6 (20.0%)</td>
<td>5 (16.7%)</td>
<td>4 (13.3%)</td>
<td>15 (16.7%)</td>
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<td>16</td>
<td>3 (10.0%)</td>
<td>4 (13.3%)</td>
<td>2 (6.7%)</td>
<td>9 (10.0%)</td>
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<tr>
<td>18</td>
<td>3 (10.0%)</td>
<td>6 (20.0%)</td>
<td>6 (20.0%)</td>
<td>15 (16.7%)</td>
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<td>21</td>
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<td>7 (23.3%)</td>
<td>4 (13.3%)</td>
<td>13 (14.4%)</td>
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<tr>
<td>23</td>
<td>2 (6.7%)</td>
<td>7 (23.3%)</td>
<td>2 (6.7%)</td>
<td>11 (12.2%)</td>
</tr>
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<td>25</td>
<td>1 (3.3%)</td>
<td>8 (26.7%)</td>
<td>4 (13.3%)</td>
<td>13 (14.4%)</td>
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<td>28</td>
<td>2 (6.7%)</td>
<td>9 (30.0%)</td>
<td>4 (13.3%)</td>
<td>15 (16.7%)</td>
</tr>
<tr>
<td>31</td>
<td>2 (6.7%)</td>
<td>7 (23.3%)</td>
<td>4 (13.3%)</td>
<td>13 (14.4%)</td>
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<td>35</td>
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<td>38</td>
<td>3 (10.0%)</td>
<td>8 (26.7%)</td>
<td>6 (20.0%)</td>
<td>17 (18.9%)</td>
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<tr>
<td>42</td>
<td>8 (26.7%)</td>
<td>7 (23.3%)</td>
<td>9 (30.0%)</td>
<td>24 (26.7%)</td>
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<td>63</td>
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<td>70</td>
<td>13 (43.3%)</td>
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<td>77</td>
<td>11 (36.7%)</td>
<td>10 (33.3%)</td>
<td>12 (40.0%)</td>
<td>33 (36.7%)</td>
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<tr>
<td>84</td>
<td>13 (43.3%)</td>
<td>11 (36.7%)</td>
<td>11 (37.9%)</td>
<td>35 (39.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>203 (27.1%)</td>
<td>194 (25.9%)</td>
<td>178 (23.7%)</td>
<td>575 (25.6%)</td>
</tr>
</tbody>
</table>

AP+AS, atovaquone/proguanil and artesunate; AS, artesunate; SP+AS+PQ, sulphadoxine-pyrimethamine, artesunate and primaquine; N, count.

Figure 2. Prevalence of positive qPCR samples by drug regimen and aggregated visit days. Samples were analysed for asexual parasite positivity (18s DNA qPCR assay) for each participant over time enrolled in the three different drug regimen groups. Percent prevalence of parasite positivity is shown over aggregated visit periods. AP+AS, atovaquone/proguanil and artesunate; AS, artesunate; SP+AS+PQ, sulphadoxine-pyrimethamine, artesunate and primaquine.
clustering by visit day (i.e. the Huber White sandwich robust variance estimator). Similar results were observed in both these models (Extended data: Supplementary Tables 1 and 2).

Molecular characterization of malaria parasites

To determine if the infections detected after drug treatment were gametocyte positive (Pfs25 RNA as the representative marker) or non-viable parasite material without active transcription (18s RNA as the representative marker), RT-PCR analysis was conducted on a subset of samples. 475 samples (26% of total representing 32 individuals) were analysed by RT-PCR after RNA extraction and cDNA synthesis.

Overall, 56.4% of the samples (348/617) within the subset assessed had concordant results for both 18s DNA and 18s RNA, while 6.32% (39/617) were negative for both (Figure 5A). 30.6% were 18s DNA positive and 18s RNA negative (i.e. implying non-viable parasites) while 6.7% (41/617) were 18s DNA negative but 18s RNA positive (implying low-levels of viable parasites). The concordance varied over time (Figure 5A). During the immediate post-treatment and follow-up period (between days 7 and 28), 18s DNA positive but 18s RNA negative parasites were more frequently observed after day 28 (chi^2 = 36.4, p<0.0005 for the comparison of visit days 7 to 28 versus after day 28) (Figure 5A).

Concordance between qPCR and gametocyte RT-PCR positivity also varied over time (Figure 5B). Prior to treatment, 3.4% (34/211) of samples were Pfs25 RNA positive. During the post-treatment period (between days 9 and 28), 9.4% (28/297) of samples were Pfs25 RNA positive, and after day 28 12.6% (63/501) of samples were Pfs25 RNA positive (Figures 5B and 5C).

Association between msp2 genotyping outcome and malaria parasite density

Genotyping was performed to differentiate where pre-treatment infection was not cleared by antimalarial treatment (i.e. “old” infection) from where participants were re-infected with new infections (i.e. “new” infection). Given the number of samples with either low density parasitaemia or different variants present where genotypes were undetected, the power
Figure 4. Determinants of qPCR positivity. Determinants of *P. falciparum* 18s DNA positivity were assessed using a multivariable logistic model. AP+AS, atovaquone/proguanil and artesunate; AS, artesunate; SP+AS+PQ, sulphadoxine-pyrimethamine, artesunate and primaquine.

Figure 5. Concordance between molecular markers of parasitaemia. Samples were analysed for asexual parasite positivity (18s DNA qPCR) and a subset of these for gametocyte positivity (Pfs25 RT-PCR) and whether they represented live parasites (18s RT-PCR) using cDNA. (a) correlation analysis between 18s DNA and 18s RNA; (b) correlation between 18s DNA and Pfs25 RNA; and (c) correlation between 18s RNA and Pfs25 RNA.
of analysis was moderate. It was therefore necessary to aggre
gate samples by time interval to test for significant variation
by drug regimen for old versus new infections. The prevalence
of “old”; “new”; or “undetected” genotypes did not dif-
fer significantly according to period of visit or drug regimen
(Table 3).

Higher levels of parasite density were associated with suc-
cess in msp2 genotyping while genotyping was highly likely to
fail or undetectable if there were low levels of parasite densities
\(p=0.0001\). Msp2 genotyping failure or undetectability was rare
above a threshold of 1000 parasites per µl (Table 4; Figure 6).

Discussion
In this study, overall parasite positive rates among study
participants with one of the three drug regimens (AP+AS, or
AS, or SP+AS+PQ), declined from 32.7% qPCR positivity
in the treatment period to 20.4% immediately post-treatment
period to 14.1% decrease in post-treatment follow-up.

RNA markers specific to gametocytes (i.e. Pfs25) and non-
specific RNA markers to detect all viable parasites (i.e. 18s)
were used to determine if the persistent parasite DNA detect-
tion despite anti-malarial drug treatment reflected viable para-
site or gametocyte. Parasite DNA was less often associated with
signals of parasite viability (i.e. 18s RNA) in the post-treatment
period compared with pre-treatment or later time periods.
Furthermore, Pfs25 positivity was infrequent following drug
treatment. Taken together, these findings suggest that gameto-
cytaemia was not a common cause for persistent qPCR parasite
positivity after treatment, and furthermore that the detection
of parasite DNA was not always linked to viable parasites,

Table 3. Association between type of infection and drug regimen.

<table>
<thead>
<tr>
<th>Old infections</th>
<th>Visit day</th>
<th>AP+AS</th>
<th>AS</th>
<th>SP+AS+PQ</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>9–28</td>
<td></td>
<td>4 (40.0%)</td>
<td>5 (50.0%)</td>
<td>1 (10.0%)</td>
<td>10 (100%)</td>
</tr>
<tr>
<td>31–84</td>
<td></td>
<td>5 (55.6%)</td>
<td>1 (11.1%)</td>
<td>3 (33.3%)</td>
<td>9 (100%)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>9 (47.4%)</td>
<td>6 (31.6%)</td>
<td>4 (21.1%)</td>
<td>19 (100%)</td>
</tr>
<tr>
<td>Pearson (\chi^2) = 3.74 P-value = 0.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>New infections</th>
<th>Visit day</th>
<th>AP+AS</th>
<th>AS</th>
<th>SP+AS+PQ</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>9–28</td>
<td></td>
<td>2 (15.4%)</td>
<td>6 (46.2%)</td>
<td>5 (38.5%)</td>
<td>13 (100%)</td>
</tr>
<tr>
<td>31–84</td>
<td></td>
<td>27 (32.5%)</td>
<td>27 (32.5%)</td>
<td>29 (47.0%)</td>
<td>83 (100%)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>29 (30.2%)</td>
<td>33 (34.4%)</td>
<td>34 (35.4%)</td>
<td>96 (100%)</td>
</tr>
<tr>
<td>Pearson (\chi^2) = 1.74 P-value = 0.42</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Undetected</th>
<th>Visit day</th>
<th>AP+AS</th>
<th>AS</th>
<th>SP+AS+PQ</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>9–28</td>
<td></td>
<td>29 (29.9%)</td>
<td>36 (37.1%)</td>
<td>32 (33.0%)</td>
<td>97 (100%)</td>
</tr>
<tr>
<td>31–84</td>
<td></td>
<td>36 (36.7%)</td>
<td>31 (31.6%)</td>
<td>31 (31.6%)</td>
<td>98 (100%)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>65 (33.3%)</td>
<td>67 (34.4%)</td>
<td>63 (32.3%)</td>
<td>195 (100%)</td>
</tr>
<tr>
<td>Pearson (\chi^2) = 1.14 P-value = 0.57</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Association between msp2 genotyping outcome and malaria parasite density.

<table>
<thead>
<tr>
<th>Parasites/µl of blood (PCR)</th>
<th>Undetected</th>
<th>Detected</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–10</td>
<td>93 (94.9%)</td>
<td>5 (5.1%)</td>
<td>98</td>
</tr>
<tr>
<td>10–10^2</td>
<td>121 (76.6%)</td>
<td>37 (23.4%)</td>
<td>158</td>
</tr>
<tr>
<td>10^2–10^3</td>
<td>52 (28.4%)</td>
<td>131 (71.6%)</td>
<td>183</td>
</tr>
<tr>
<td>10^3–10^4</td>
<td>4 (2.8%)</td>
<td>141 (97.2%)</td>
<td>145</td>
</tr>
<tr>
<td>10^4–10^5</td>
<td>1 (0.8%)</td>
<td>122 (99.2%)</td>
<td>123</td>
</tr>
<tr>
<td>10^5–10^6</td>
<td>2 (2.2%)</td>
<td>91 (97.9%)</td>
<td>93</td>
</tr>
<tr>
<td>10^6–10^7</td>
<td>0 (0.0%)</td>
<td>6 (100%)</td>
<td>6</td>
</tr>
<tr>
<td>10^7–10^8</td>
<td>0 (0.0%)</td>
<td>5 (100%)</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>273 (33.7%)</td>
<td>538 (66.3%)</td>
<td>811</td>
</tr>
</tbody>
</table>
raising the possibility that DNA detection methods might be positive even in the absence of live parasites. These findings are consistent with a study involving travellers treated for malaria in Sweden who had no risk of reinfection where parasite DNA was detectable by species-species qPCR up to the day 42 after treatment.

The persistence of pre-vaccination DNA positivity complicates the use of PCR detectable infections as an endpoint for vaccine efficacy as it has the potential to result in an underestimation of vaccine efficacy. This is particularly important in case of pre-erythrocytic vaccines where the vaccine cannot conceivably protect against pre-vaccination blood-stage parasitaemia. Where an effect of a pre-erythrocytic vaccination is seen in a randomized trial, this may be taken as evidence of vaccine effect, but where no effect is seen one cannot rule out that this may be the result of incomplete pre-vaccination asexual parasitemia clearance and suppression of new infections in both controls and vaccines.

Compared with AP+AS, participants who were treated with AS or SP+AS+PQ were more likely to be qPCR parasite positive during the post-treatment follow-up period. Since parasite rates immediately post-treatment (between days 9 and 14) were similar for all three drug regimens, we conclude that the difference between the post-treatment follow-up period (days 16 and 28) are on balance more likely to be due to variations in reinfection rates between the drug regimens rather than variations in clearance rates. However, many samples were positive for low parasite densities, and it was often not possible to genotype the infections in order to distinguish recrudescence from reinfection to confirm this. Furthermore, although these differences were statistically significant, there is still significant DNA positivity after treatment with AP+AS, hence even this drug regimen is far from ideal for Phase IIb studies with PCR positivity as an endpoint.

It is unlikely that artesunate tolerance or resistance is relevant to these findings since the genetic polymorphisms associated with artesunate resistance have not been found in surveys in Coastal Kenya. The long-term suppression of reinfection would be consistent with the previously described long-lasting effect of atovaquone. The associations between qPCR positivity and \textit{msp2} genotyping outcome provides a measure to what level of parasite density one is able to resolve parasite \textit{msp2} genotypes.

Studies using DNA-based qPCR positivity for malaria parasites as an endpoint for vaccine efficacy have been done in non-endemic areas such as the Netherlands, and The Gambia where malaria is more markedly seasonal than in Kenya, and it is possible that this form of surveillance is easier to interpret in settings where a long dry season results in less frequent asexual parasite positivity prior to the start of monitoring.

**Limitations**

The sample size was limited to 30 participants per drug regimen. This limits power, particularly when infections are divided into new infection versus recurrence of previous genotypes. Genotyping undetectability was common at low parasite densities,
making it difficult to determine if the infection detected post-treatment was new or old. The study was undertaken in a single site and a single transmission season. It is possible that delayed parasite clearance would be less problematic in a site where transmission is minimal outside a malaria season that occurs after randomization, where fewer participants would have high density parasitaemia at the start of monitoring.

Conclusion
There was an overall significant reduction in the prevalence of malaria parasites by qPCR between day 9 and day 14 following treatment of the study participants with either of the three drug regimens, though it was surprising that substantial levels of qPCR parasite positivity were seen after day 9, and these were often due to parasite genotypes that had been present prior to treatment. Although AP+AS was less prone to this problem than the other regimens examined, it also may have inhibited the acquisition of new infections. More robust assessments of vaccine efficacy in small-scale Phase IIb trials may be obtained in controlled human malaria infection studies where the exposure is experimentally controlled and volunteers can be selected for falciparum DNA-based PCR monitoring prior to exposure to infection.

Data availability
Underlying data
Harvard Dataverse: Replication Data for: Comparing Drug Regimens for Clearance of Malaria Parasites in Asymptomatic Adults using PCR in Kilifi County, Kenya: an open-label randomised controlled clinical trial (MalPaC), https://doi.org/10.7910/DVN/HJHSIC.

This project contains the following underlying data:
- MALPAC_merged_20190114_unblinded.tab
- NKhaemba_ParasiteClearance_Codebook.pdf
- NKhaemba_ParasiteClearance_doFile.do
- NKhaemba_ParasiteClearance_Readme.txt

Extended data

This project contains the following extended data:
- Supplementary table 1: Determinants of PCR positivity using multilevel logistic regression
- Supplementary table 2: Determinants of PCR positivity using logistic regression

Reporting guidelines
Harvard Dataverse: Consort checklist for: ‘Comparing Drug Regimens for Clearance of Malaria Parasites in Asymptomatic Adults using PCR in Kilifi County, Kenya: an open-label randomised controlled clinical trial (MalPaC)’. https://doi.org/10.7910/DVN/CVZMNV.

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Acknowledgements
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http://www.doi.org/10.7910/DVN/HHD65G


StataCorp: Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC, 2017. Reference Source


http://www.doi.org/10.7910/DVN/JHHSIC