ARTICLE

Factors determining the occurrence of submicroscopic malaria infections and their relevance for control

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Malaria parasite prevalence in endemic populations is an essential indicator for monitoring the progress of malaria control, and has traditionally been assessed by microscopy. However, surveys increasingly use sensitive molecular methods that detect higher numbers of infected individuals, questioning our understanding of the true infection burden and resources required to reduce it. Here we analyse a series of data sets to characterize the distribution and epidemiological factors associated with low-density, submicroscopic infections. We show that submicroscopic parasite carriage is common in adults, in low-endemic settings and in chronic infections. We find a strong, non-linear relationship between microscopy and PCR prevalence in population surveys ($n=106$), and provide a tool to relate these measures. When transmission reaches very low levels, submicroscopic carriers are estimated to be the source of 20–50% of all human-to-mosquito transmissions. Our findings challenge the idea that individuals with little previous malaria exposure have insufficient immunity to control parasitaemia and suggest a role for molecular screening.

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Measuring the prevalence of malaria infection in population surveys underpins surveillance and control of the parasite. During more than a century of malaria research, parasite infection has been assessed by light microscopy of blood films. This wealth of data is widely used to understand malaria epidemiology, to monitor and inform control strategy[1], to map the geographical distribution of malaria over time[2] and to aid development of mathematical models. Rapid diagnostic tests (RDTs) based on antigen detection are now also used for prevalence surveys. However, both techniques have limited sensitivity. Molecular detection techniques for malaria[3] have a much higher sensitivity and are increasingly revealing the widespread presence of infections with parasite densities below the detection threshold of either microscopy or RDTs. These results fundamentally challenge our current view of malaria epidemiology and burden of infection.

In a previous systematic review and meta-analysis we found that microscopy misses on average half of all *Plasmodium falciparum* infections in endemic areas compared with PCR[4]. There was high variability between surveys and transmission settings. It remains unclear what factors cause this variation in levels of submicroscopic infections, and to what extent such infections are relevant to current efforts to control and eliminate the parasite. From a clinical perspective, low-density infection has been associated with mild anaemia[5] and adverse effects during pregnancy[6], but rarely causes acute symptoms. Nevertheless, the public health importance of low-density infections may be significant, as experiments have shown that mosquitoes feeding on individuals who are parasite-negative by microscopy can become infected with malaria[7,8].

The probability of detecting malarial infection is a function of the density of parasites and the volume of blood examined. Parasite densities in the peripheral blood fluctuate considerably over the course of any single *P. falciparum* infection and may dip under the microscopic detection threshold[9] due to sequestration during the second half of the 48 h life cycle and varying effectiveness of the host's immune response. The volume of blood examined during microscopy slide-reading, if 100 high-power fields are screened, is 0.1–0.25 μl (refs 10–12), whereas for PCR detection DNA is extracted from 5 to 100 μl in most commonly used protocols. On the basis of these volumes, the theoretical detection limit for standard thick film microscopy is approximately 4–10 parasites per μl, and for PCR it is 0.01–0.2 parasites per μl. In practice, a low number of parasitized red blood cells in a sample is often not sufficient to enable detection due to technical factors such as loss of parasites during staining of microscopy slides[10,13] or use of single versus nested PCR protocols. Calibration against cultures with known parasite densities has shown realistic detection limits of 10–100 parasites per μl for microscopy[14] and 0.05–10 parasites per μl for various PCR assays[15,16].

From the perspective of control agencies aiming to reduce transmission, the most important question is to what extent do submicroscopic parasite carriers, who are missed during routine surveys, contribute to sustaining transmission? To become infected with malaria, *Anopheles* vectors need to take up a minimum of one male and one female gametocyte in a 2- to 3-μl bloodmeal. There is still a considerable probability of this happening at parasite densities that will often be missed by microscopy (for example, 1–10 parasites per μl), both according to mathematical theory and data[17], and an aggregated distribution of parasites in the blood may assist transmission at very low densities[18]. During the scale-up of malaria control, public health agencies must decide what screening tools to use in different populations and whether submicroscopic carriers are a priority for intervention[19]. With sufficient resources, submicroscopic parasites could be detected in active screening programmes[20] and included in evaluations where they may alter estimates of how interventions impact the prevalence of infection. Both from a biological and a public health perspective, it is important to understand where and when submicroscopic carriage is mostly likely to occur. Here we compile and analyse epidemiological data sets to assess firstly the prevalence of submicroscopic parasite carriers, and secondly which factors cause these carriers to be more numerous in some areas and population groups. We explore the roles of immunity, antimalarial treatment, level of malaria endemicity and technical test performance. On the basis of 106 PCR prevalence surveys, we develop an analysis tool to estimate how prevalent such carriers are likely to be in any given area. We estimate the contribution of submicroscopic parasite carriers to the onward transmission of malaria by combining survey data with human-to-mosquito transmission studies.

Results

Submicroscopic parasitaemia across the endemicity spectrum. We compiled survey data in which *P. falciparum* prevalence was measured by both microscopy and by PCR in the same individuals through updating a previous systematic review[4]. One hundred and six surveys met our inclusion criteria for analysis, taking place in endemic populations within a defined geographic area where participants were not selected according to malaria symptoms or test results, and where nested PCR or equivalent was used for parasite detection (see also Methods and Supplementary Table S1). Submicroscopic carriers were defined as those individuals with infections detected by PCR but not by microscopy. The specificity of microscopy relative to PCR is very high (98.4% on average[4]), and given infrequent reporting of specificity in the included studies we assume in our analysis that slide-positive results are also PCR-positive. Microscopy detected, on average, 54.1% (95% confidence interval (CI), 50.3–58.2%) of all PCR-detected infections across the 106 surveys, but this sensitivity varied widely (Fig. 1a) as in previous analysis[4]. Regression analysis showed that the PCR prevalence of infection has a strong linear relationship with microscopy prevalence on the log odds scale (Fig. 1a). Stratifying by age group improved the fit to the data with microscopy sensitivity being higher in children only (<16 years), lowest in adults (16 + years) and at an intermediate level when both adults and children were included in the survey (Fig. 1a and b). The best estimate of PCR prevalence is obtained from microscopic slide prevalence as follows (Fig. 1a and b):

$$\text{log odds PCR prevalence} = 0.954 + 0.868 \times \text{log odds slide} - \text{prevalence (all age groups)}$$

$$\text{log odds PCR prevalence} = 0.755 + 0.984 \times \text{log odds slide} - \text{prevalence (children only)}$$

$$\text{log odds PCR prevalence} = 1.106 + 0.810 \times \text{log odds slide} - \text{prevalence (adults only)}$$

where prevalence is a proportion (on the scale 0 to 1), log odds $= \log_e(\text{prevalence}/(1-\text{prevalence}))$ and prevalence $= e^{(\text{log odds})}/(1 + e^{(\text{log odds})})$.

As the effect of age on the sensitivity of microscopy was significant, and most surveys ($n = 86$) included both children and adults, we focussed on these studies with a wide age range for further analysis. We assessed the ability of the regression model to predict PCR prevalence from slide prevalence using a leave-out-one cross validation procedure. The correlation coefficient between
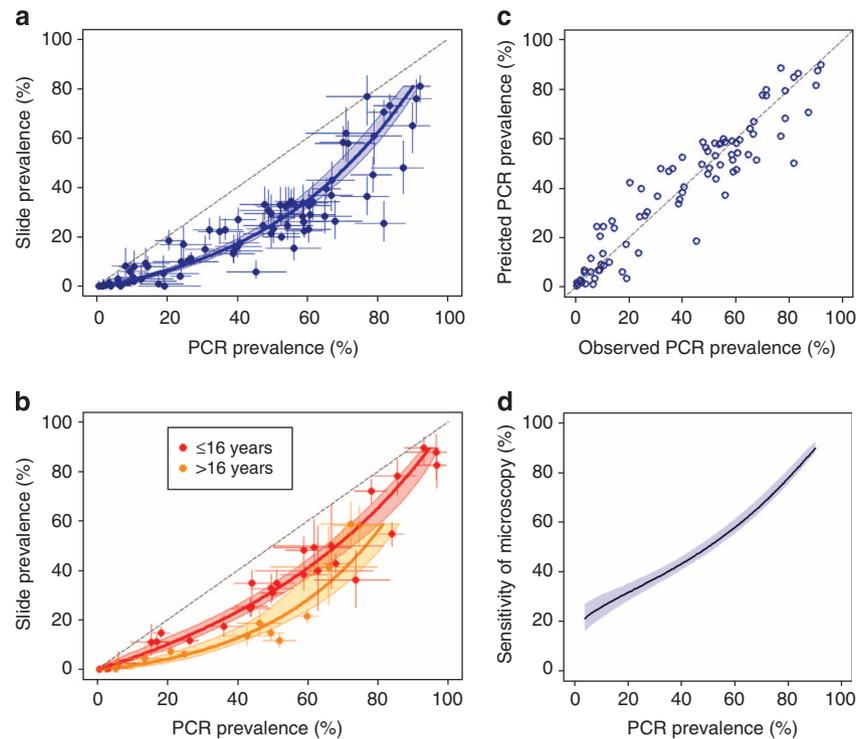
Figure 1 | Prevalence of infection by PCR versus microscopy in 106 prevalence surveys and model fits. Bayesian Markov chain Monte Carlo methods were used in model fitting to allow for measurement error in both microscopy and PCR prevalence measures[50]. Posterior medians are shown. (**a**) Prevalence data with 95% CI from 86 surveys containing both adults and children, and fitted model (blue line) with 95% credible interval of the mean (light blue area). The model fitted was a linear relationship on the log odds scale. (**b**) Age-specific prevalence with 95% CI for survey data containing children only (<16 years, red dots, $n=28$) or adults only ($\geq$16 years, orange dots, $n=13$). Fitted models (lines) with 95% CI (shaded areas) were obtained using the same methods as in **a**. (**c**) Correlation between observed and model-predicted PCR prevalence in the all-age surveys (**a**), obtained using leave-one-out cross-validation. Correlation coefficient $=0.941$. (**d**) Estimated average sensitivity of microscopy and 95% credible interval of the mean in the all-age surveys according to underlying PCR prevalence.

observed and predicted values was 0.941, indicating good agreement (Fig. 1c). Using the fitted model, we provide a supplementary spreadsheet tool (Supplementary Software) to estimate PCR prevalence in areas where only slide-prevalence data are available and vice versa, using the equations above, with 95% uncertainty bounds on the prediction that take into account both the uncertainty in our fitted model and the sample size of the user-entered prevalence measure (see Methods). Our tool applies to cross-sectional population surveys with participants from all age groups, and readers should be cautious about using this tool if their data is from a different type of study population. Further details are provided in the Supplementary Software.

Our fitted model indicates that the numbers of submicroscopic carriers as a proportion of the total infected population show a striking trend with changing transmission intensity, decreasing from an average of between 70 and 80% in areas with low PCR prevalence to 20% in the highest prevalence areas (Fig. 1d; see also ref. 4). In the following sections we examine the evidence for the potential role of several host and parasite factors in giving rise to low-level parasitaemia and whether these could drive the observed trend over the transmission intensity spectrum.

Submicroscopic parasitaemia in malaria-naive individuals. Malaria therapy studies, in which laboratory-infected patients were monitored closely over time, are an important source of information on the within-host dynamics of malaria parasites in non-immune individuals[21,22]. We examined longitudinal data from 89 individuals infected with *P. falciparum*, who had not

previously had malaria and were not treated with anti-malarials at any point. Although only microscopic detection was available at the time, blood slides were taken continually even after a patient had become slide-negative. This showed that most patients experienced a phase when parasite densities fluctuated under and over the microscopic detection threshold, and allowed periods of submicroscopic infection to be identified. The time since infection was an important determinant of parasite density and detectability. The probability of detecting parasites in patients who had not yet had their last episode of slide-positive parasitaemia, that is, they were still infected, showed a clear decline over time (Fig. 2a), dropping to ~0.85 after 50 days, and less than 0.25 after 200 days (Fig. 2a). The fact that parasite densities are reduced below the microscopic detection threshold is likely to reflect an anti-parasitic immune response developing in these individuals as they experience their first malaria infection.

Age and acquired immunity. Increasing age has been clearly linked to lower parasite densities[12,23], and our analysis above also finds a significantly higher proportion of submicroscopic carriers among surveys of adults compared with children (Fig. 1b). For a more detailed analysis of the role of age, we extracted data from 11 studies where prevalence by microscopy and PCR was broken down for three or more age groups. The data shows a general trend for more infections to be submicroscopic in older children and adults compared with young children (Fig. 3). In linear regression analysis, the proportion of all infections detected by microscopy decreased by 0.7% (95% CI, 0.2–1.2%) per year of age
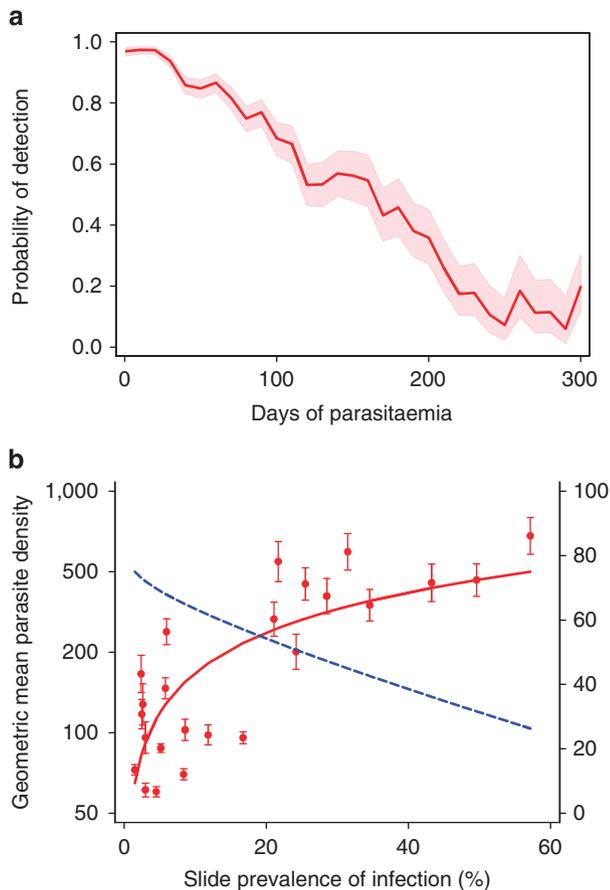
a

b

Figure 2 | Trends in parasite densities within-host and across different transmission settings. (**a**) Average probability of microscopic detection over the course of a single *P. falciparum* infection in 89 individuals, among those who had not yet had their last episode of patent parasitaemia (data from malaria therapy studies [21,22]). None were treated nor had a record of previous infection. Results are shown up to 300 days (some patients had longer infections, but there were few data points after this time). Points and CIs were calculated in 10-day time bands. (**b**) Relationship between slide prevalence and parasite density among infected individuals in 24 Tanzanian villages (data from Drakeley *et al.*[24] red points = data with 95% CI, red line = linear regression fit on log scale), and the estimated percentage of infections which are submicroscopic in all age groups based on the model in Fig. 1a (blue dashed line).

in the pooled age-specific data. This almost certainly represents greater immunity in older individuals, likely due to both cumulative exposure and a more developed immune system.

Given the role of immunity in suppressing parasite densities, individuals living in areas of high transmission with greater exposure might be expected to have less readily detectable infections. However, the between-area comparison of 106 surveys shows the opposite, with submicroscopic carriers predominating in areas of lower transmission (Fig. 1d). In the detailed age-specific data (Fig. 3), age and underlying population PCR prevalence remain significant predictors of microscopy sensitivity in linear regression analysis after mutual adjustment ($P<0.001$ for both factors). There was no evidence that increasing age had a more pronounced effect on the proportion of submicroscopic infections in areas of higher endemicity, where a larger age-dependent difference in acquired immunity may be expected. Indeed, there was a borderline significant association for a smaller effect of age in higher transmission settings ($P=0.055$). These data suggest that other factors override the effects of population

immunity to produce on average more detectable, high-density infections in high-transmission areas. To further quantify the relationship between parasite densities and transmission intensity, we examined data from 24 Tanzanian villages where transmission intensity varied across from less than 1 infectious bite per person per year, to ∼100 and was reflected by slide prevalence ranging from 2 to 57%[24]. Parasite densities assessed by microscopy show a clear trend of increasing with increasing transmission intensity (Fig. 2b). This is in line with the trend showing more readily detectable infections where transmission in high and with previous observations[25]. As more quantitative PCR data becomes available, it should be possible to confirm whether this relationship holds when submicroscopic densities are taken into account.

Antimalarial treatment. When many of the PCR prevalence surveys in the review data were carried out, there was spreading resistance to the most commonly used antimalarial drugs available at the time, chloroquine and sulphadoxine-pyrimethamine. These partially effective treatments could have been a contributing factor to the levels of submicroscopic carriage in the data through allowing persistent, low-level infections[26]. We contrasted national first-line treatment policies at the time of the studies in our review, and found that 21 countries recommended artemisinin combination therapies and 85 recommended non-artemisinin-based treatments such as sulphadoxine-pyrimethamine. There was no significant difference in the proportion of infections that were submicroscopic, in surveys carried out when artemisinin combination therapies were the first-line treatment ($P=0.186$), which should have been fully effective at clearing parasitaemia at the times and locations that the surveys were done. Nor was there any trend in changing microscopy sensitivity according to the year the study was carried out ($P=0.868$), as might have been expected because of increasing drug resistance over time. These results give no evidence for an important role for suboptimal treatment in explaining the distribution of submicroscopic infections between populations.

Parasite detection methods. Variation in microscopy and PCR methodology is known to affect their performance and sensitivity. In the systematic review data, there were borderline significant increases in microscopy sensitivity with the volume of blood examined, as indicated by the number of microscopic fields examined ($P=0.043$) or the number of leucocytes counted ($P=0.046$; see also ref. 4). There was no association of microscopy sensitivity relative to PCR with the other methodological variables we examined in our analysis: the volume of blood used for DNA extraction (information available for 27 studies), the PCR protocol (Snounou *et al.*[15] or McNamara *et al.*[16] versus other protocols, $n=106$ (see also ref. 4)) or the quantity of extracted DNA used in the PCR ($n=23$). Some of these variables are known to be important from previous studies, so the number of surveys with the relevant data available may have been too small to detect any associations, or other variation between studies could have obscured them. Indeed, variation between laboratories has been found to be more important than variation between protocols for PCR performance[27]. Using dried blood collected on filter paper for PCR analysis also did not affect sensitivity, in line with previous findings[27].

PCR can be subject to contamination, giving false positives and potentially overestimating submicroscopic carriage[28]. Negative controls should be included during every PCR. However, there was no association between studies reporting use of a negative
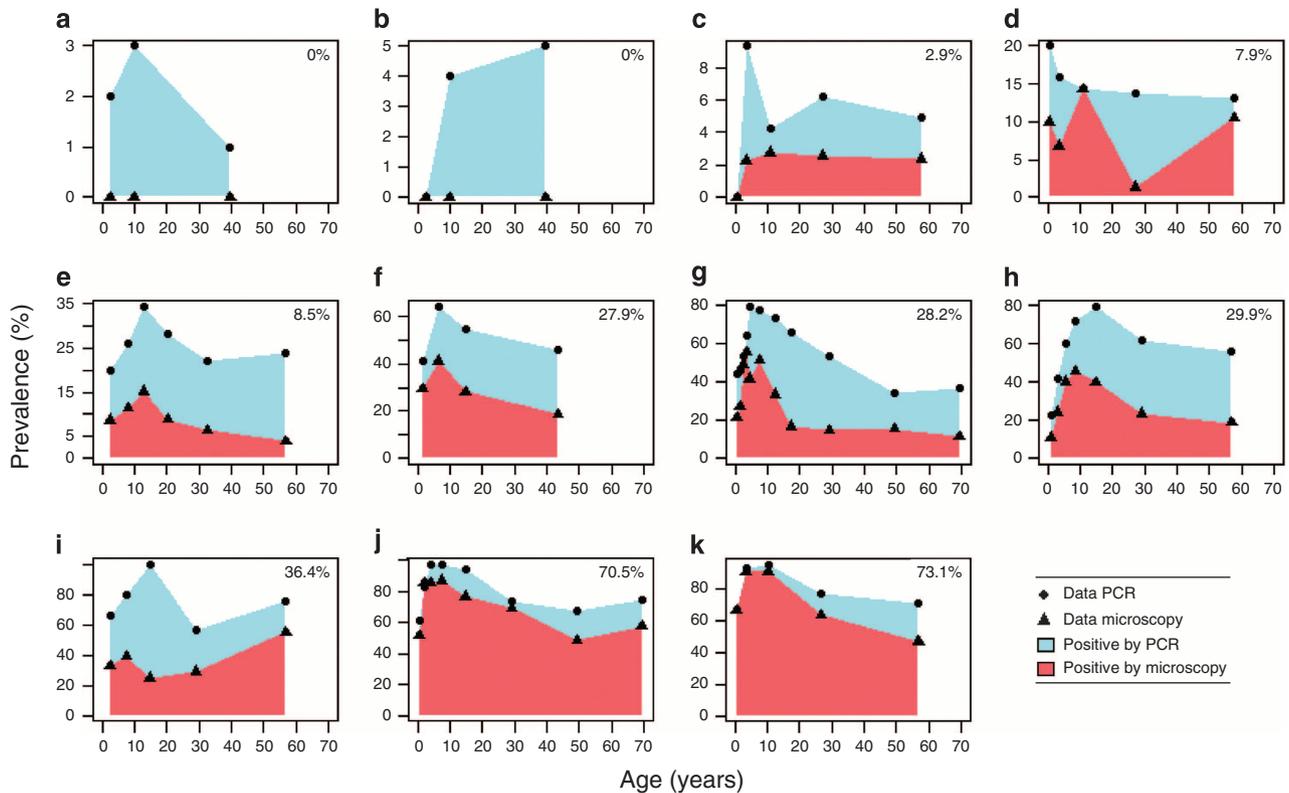
Figure 3 | Age-specific microscopy and PCR prevalence. Eleven studies reported prevalence for three or more age groups (references **a–b**[51], **c–d**[52], **e**[53], **f**[54], **g**[55], **h**[56], **i**[57], **j**[58], **k**[59]). Microscopy prevalence in the whole study population is shown in the top-right corner of each panel.

Table 1	Infectiousness of submicroscopic parasite carriers versus slide-positive carriers.				

Study	Country	N people	N mosquitoes	% Mosquitoes infected, slide-positives	% Mosquitoes infected, submicroscopic infections
Ouedraogo 2011 (ALO, unpublished data)	Burkina Faso	260	10,244	12.8	3.2
Jeffery and Eyles[29]	USA	95	10,096	20.3	2.0
Coleman et al.[30]	Thailand	237	9,210	0.4	0.2*
Young et al.[7]	USA	124	6,600	8.4	0.5*

Proportion of mosquitoes infected during human-to-mosquito transmission studies. In two of these studies, the prevalence of submicroscopic carriage among microscopy-negatives was not assessed and therefore this was estimated using the relationship in Fig. 1a.
*Prevalence of submicroscopic carriers estimated.

control and the proportion of submicroscopic infections as previously found[4]. In addition, assuming any amount of PCR contamination (that is false-positive PCR results) considerably worsened the fit of the model; for example, the deviance information criterion for the model with 5% contamination was 175 larger than the model with no contamination, giving no support for contamination being a significant cause of apparent submicroscopic carriage.

Implications for control programmes and elimination. To assess the contribution of submicroscopic infections to malaria transmission, we reviewed human-to-mosquito transmission studies and identified four experiments which included individuals who tested negative for malaria by microscopy in malaria-endemic areas (defined as no asexual parasites nor gametocytes; refs 7,29,30 and ALO, unpublished data; Table 1). Further studies tested the infectiousness of individuals with asexual parasites but no gametocytes; however, we focussed on carriers who would not be identified at all during routine, microscopy-based surveys.

Molecular studies have shown that most people with asexual parasites also carry gametocytes[17,31], although they are more rarely detected by microscopy because they occur at a lower density[8]. The results varied from showing an approximate 2-fold to a 16-fold reduction in infectivity in submicroscopically infected versus microscopy-positive individuals. The estimate of a two-fold reduction came from a study where an unusually low proportion of mosquitoes were infected in total; only 0.4% of those fed on slide-positives, increasing the uncertainty of the estimate[30]. In the analysis, therefore, we used the other three estimates that show a 4- to 16-fold reduction in infectivity (Table 1).

We estimated the contribution of the submicroscopic reservoir to sustaining malaria transmission, that is, the proportion of mosquito infections originating from submicroscopic carriers. We combined the average prevalence of submicroscopic infection across different malaria endemicities from the survey data analysis (Fig. 1a) with each of the three studies on infectiousness (Table 1).

Two of the infectiousness studies[7,29] suggest that submicroscopic carriers are relatively unimportant, except when transmission
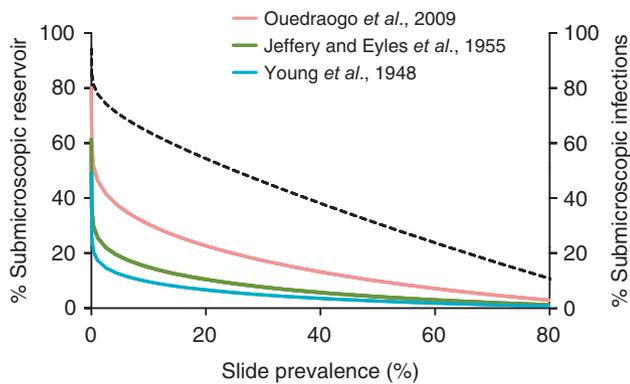
Figure 4 | Estimated contribution of submicroscopic infections to the infectious reservoir. Percentage of mosquito infections originating from submicroscopic carriers, according to the observed slide prevalence of infection. Estimates are based on results from Fig. 1a and measures of infectiousness from three studies (refs 7,21 and ALO, unpublished data). The estimated % of all infections which are submicroscopic from Fig. 1a is shown as a black dashed line.

Figure 5 | Hypothesized within-host dynamics of parasitaemia. Schematic showing hypothesized patterns of parasitaemia in a single host in low- and high-transmission settings, showing infection times (arrows) and periods of submicroscopic parasitaemia (grey areas). Grey lines indicate detection limits of microscopy and PCR.

is very low (Fig. 4). Submicroscopic carriers are estimated to be the source of over 20% of mosquito infections only where slide prevalence is under 4%, for the first of these two studies,[29] or under 0.5% for the second study.[7] The third study (ALO, unpublished data) suggests submicroscopic infections are the source of over 20% of mosquito infections where slide prevalence is up to 24%, and as much as 50% of mosquito infections in very low-transmission areas (slide prevalence <0.5%). This analysis has limitations and additional estimates on the infectiousness of microscopic and submicroscopic parasite carriers are required, taking into account that the infectiousness of submicroscopic parasite carriers may vary between settings. Despite these uncertainties, all results indicate that submicro-

scopic infections are important contributors to transmission in areas with low or very low transmission intensity (under ~0.5%) and they may be important when slide prevalence is up to 24%, but contribute considerably less to transmission in areas of high transmission intensity.

Discussion

Our analysis shows that submicroscopic infections are likely to be important in sustaining transmission in areas where slide prevalence is low (<10–20%). For example, in an area where slide prevalence is 3%, submicroscopic infections are present in a further 8% of the population and, our calculations suggest, are responsible for 14–40% of all mosquito infections (Fig. 4). Submicroscopic carriers will become increasingly important as current control programmes continue to successfully reduce transmission intensity. We find that the prevalence of submicroscopic carriage can be estimated relatively precisely for a given population from microscopic slide prevalence using a simple model. As PCR methods are not yet widely available in the field, such a relationship can provide a useful approximate guide to underlying submicroscopic carriage in areas where only slide prevalence is available. Control and elimination programmes may wish to consider using molecular methods to identify infected individuals; our results warn against a relaxation of control efforts once prevalence of infection appears low according to microscopy. Molecular screening for parasites will be facilitated by the development of newer, field-adapted molecular tests[32].
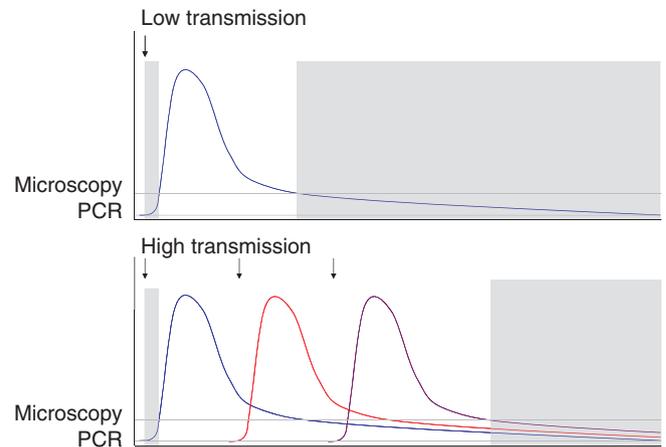
The common assumption that individuals with little previous exposure to malaria have a limited immune response against parasites and usually experience high-density infections is challenged by molecular data. The prevalence of submicroscopic infections in both low-transmission areas and in children suggests that a parasite-suppressing immune response develops more efficiently than commonly assumed. In malaria therapy patients, a common pattern is that initial high parasite density is followed by a long phase of low-density parasitaemia, which often drops below the microscopy detection threshold. There is evidence that in semi-immune individuals living in endemic areas, new infections similarly cause a phase of higher parasitaemia followed by a chronic low-density phase[23,33], although this is difficult to confirm as multiple infections often overlap and most current genotyping methods do not show the contribution of each clone to total parasite density. However, the arrival of new parasite genotypes in a host has been linked to clinical attacks, even in the presence of asymptomatic infection with other parasite clones, reflecting a rise in parasite density[34]. Seasonal patterns also indicate a rise in parasite density during the rainy season when rates of infection are high, even when there is little seasonal variation in parasite prevalence.[25]

On the basis of these observations, combined with malaria therapy data, we hypothesize that frequent infection and super-infection in high-transmission areas increase the average parasite density in infected individuals, whereas in lower transmission areas infections are on average older and more likely to have reached a submicroscopic phase. Figure 5 shows a schematic representation of our hypothesis. Although immunity clearly has some role in suppressing parasite densities to low levels (as shown by the age patterns of microscopy and PCR prevalence in Fig. 3), it cannot explain the varying rates of submicroscopic carriage across endemic settings. We hypothesize that the average time since last being infected overrides the effects of immunity in determining the low rate of submicroscopic parasitaemia in high-endemic settings (Fig. 5). The reason that new infections result in higher parasite density and clinical attacks is not fully understood, but suggests hosts may have only partial cross-immunity against different parasite clones. Being infected with multiple clones has also been correlated with higher parasite density[35]. This could simply reflect a higher probability of having a recent infection, or may alternatively demonstrate an

independent effect on parasite density. For example, host immune systems may be overwhelmed by the additional number or variety of parasites, or parasites may facultatively increase their growth rates in the presence of competing clones[36].

If our hypothesis is correct, the prevalence of submicroscopic infections in an area may reflect recent dynamics of transmission and indeed levels of control interventions[37]. A high proportion of submicroscopic infections could indicate a recent decrease in transmission. This is consistent with data from northern Tanzania showing high (33%) prevalence of almost entirely submicroscopic infections during a time of declining transmission[38]. Such dynamics may occur when the local transmission potential is stably reduced, for example, by ongoing vector control. However, submicroscopic infections can be sufficient to restart transmission in the right conditions. For example in Sudan, slide prevalence declines to zero during the long dry season but some PCR-positive cases remain[26], who are presumed to explain the resurgence of transmission with the next rainy season once there are sufficient vectors. Therefore, a successful control programme should not be relaxed while submicroscopic infections persist.[39]

There may be additional factors associated with submicroscopic carriage that we could not examine in our current analysis. Haemoglobinopathies and other human polymorphic traits, which protect against malaria, are associated with lower parasite density[40]. However, such traits are generally more common when malaria transmission is high, which would not explain the lower proportion of submicroscopic infections in these areas as observed in our review data. Parasites may also have varying genetic propensities to reach particular densities when infecting hosts, for example, through different multiplication rates[41]. Genetic diversity in an area may affect parasite densities. Molecular studies have shown that low genetic diversity is linked with low transmission intensity[42], suggesting that after people are infected once, subsequent infections contain antigenically very similar parasites and may therefore be easier to control. There is also a short submicroscopic phase at the beginning of an infection during initial blood-stage parasite multiplication. In adults in endemic areas, this multiplication rate is over three-fold lower than in non-immune hosts[43], which could contribute to the age patterns of parasite detectability. Although our analysis did not find a role of partially effective anti-malarial treatment in enhancing low-level parasitaemia, there is evidence from Sudan that chronic submicroscopic infections were associated with recent chloroquine treatment during a time of increasing parasite resistance[44]; further molecular follow-up data from clinical trials would provide clarification on this.

We did not analyse the relationship between RDT and PCR prevalence because of a paucity of surveys reporting both measures. However, the development of large, publicly available prevalence data sets[45] will provide the opportunity to characterize the relative sensitivity of microscopy and different RDT types, and from there it will be possible to get a clearer idea of RDT–PCR prevalence relationships. In clinical settings, the sensitivity of standard RDTs is generally high but their detection limits are around 100 parasites per μl[46]; therefore, they will be generally somewhat less sensitive than microscopy in population surveys, where parasite densities are lower. There are also known to be malaria infections with very low density missed by both microscopy and PCR[47].

The ubiquity of submicroscopic carriers in low-transmission sites indicates that assessing parasite carriage with molecular tools will be critical in monitoring malaria elimination programmes, where microscopy measures are insufficient to assess the scale of public health measures required. Identifying these carriers and their characteristics could also shed new light on ecological or

sociodemographic risk factors for malaria in areas where most infections are submicroscopic. The evolutionary advantage to the parasite of these chronic, low-density infections is clear, particularly as mosquitoes can still become infected. In some areas with highly seasonal or sporadic vector populations, the ability to create chronic infections in the human host may be essential for parasite survival[26]. This advantage also extends to the human host, as such low-density infections cause minimal disease and can maintain immunity[48]. The increasing quantity of emerging molecular data will continue to enlighten our understanding of human-parasite interactions and their co-evolution.

Methods

Systematic review update and analysis. We compiled malaria prevalence data where both microscopy and PCR methods had been used to determine infection status, through updating a previous systematic review using the same methods[4]. We searched from January 2009, when the previous review was completed, to the 1 November 2011, retrieving 636 records using the search terms 'malaria' and 'PCR', and through searching reference lists in the articles retrieved. Surveys were included if they took place in endemic populations within a defined geographic area, and were excluded if participants were selected according to malaria symptoms or test results, if the survey was gametocyte-specific, or if there was a large-scale intervention before data collection. We also excluded surveys of pregnant women, which are a special case and have been reviewed elsewhere[49]. After screening titles and abstracts, we retained 73 for full-text evaluation. Fifteen of these were included and yielded 51 microscopy and PCR prevalence survey data pairs. Together with the previous systematic review results, this gave a total of 122 prevalence surveys. We restricted analysis to 106 surveys, which used a nested or semi-nested PCR or a PCR with an additional ligase detection reaction step[16], to aim for equivalent sensitivity, and those which provided the age range of survey participants. We extracted information on covariates with potential influence on microscopy sensitivity, including the age range of participants and details of microscopy and PCR procedures.

We used the method described by Sharp et al.[50] to fit a linear relationship to the log odds of PCR and microscopy prevalence data:

$$\Omega_{\text{P}i} = \Omega_{\text{M}i} + \delta_i \tag{1}$$

$$\delta_i = \delta_i' + \beta_0(\Omega_{\text{M}i} - \overline{\Omega_{\text{M}}}) \tag{2}$$

where $\Omega_{\text{P}i}$ is the log odds of PCR prevalence in trial i, $\Omega_{\text{M}i}$ is the log odds of microscopy prevalence, δ is the log odds ratio (OR) of PCR to microscopy prevalence, $\overline{\Omega_{\text{M}}}$ is the mean log odds of microscopy prevalence across trials, δ' is the expected log OR PCR:microscopy prevalence when the log odds of microscopy prevalence is equal to the mean across trials, and β_0 is a regression coefficient. Any prevalence survey showing zero positive results by either microscopy or PCR was assumed to have 0.5 positive individuals, that is, half the detection limit, so they could be included in the analysis. The specificity of microscopy relative to PCR is very high (98.4% on average[4]), so we assumed in our analysis that all slide-positive results are also PCR-positive. The model was fitted using Bayesian Markov Chain Monte Carlo techniques in Winbugs to allow for the different sample sizes and thus sampling variation in each survey. Each observed prevalence value is assumed to be drawn from a binomial distribution with the sample size of the survey. The log OR of microscopy: PCR prevalence was allowed to vary between surveys, with both the log OR and the log odds of microscopy prevalence assumed to be normally distributed. Uninformative priors were used. We assessed the predictive value of the fitted model using a leave-out-one cross validation procedure. Using the best fitting model, we developed a spreadsheet tool, which converts user-entered slide-prevalence data to PCR prevalence, or vice versa. Uncertainty bounds on the prediction were calculated by applying the delta method to the posterior distributions of fitted parameters and the Bayesian CI of the prevalence data entered by the user.

As an amplification technique, any contamination of PCR could have a significant effect on the model; therefore, we tested it as a potential covariate. The observed PCR prevalence $I_{\text{P}i}$ was assumed to contain a constant proportion of contaminated samples c, that is, false positives. We fitted the model above with the following modification:

$$I_{\text{P}i} = \hat{I}_{\text{P}i} + (1 - \hat{I}_{\text{P}i})c \tag{3}$$

where $\hat{I}_{\text{P}i}$ is the PCR prevalence that would be observed in the absence of contamination, and $\Omega_{\text{P}i}$ in equation 1 above becomes the log odds of $\hat{I}_{\text{P}i}$ rather than the log odds of $I_{\text{P}i}$. We tried both fitting and fixing the proportion of samples that are contaminated. When this was fixed, we used a value of 5% based on reports from laboratories[28]. We additionally examined whether several other variables, particularly variations in the PCR and microscopy technical methods, influenced microscopy sensitivity using standard random-effects meta-analysis and meta-regression.

Malaria therapy data. We analysed data from patients infected with the El-Limon, McLendon or Santee-Cooper (gametocyte-producing) strains of *P. falciparum*[21,22]. We excluded patients who were treated or reported a previous infection, and data from the day after the last microscopic detection of parasites for each patient. Infectiousness of submicroscopic relative to microscopic cases was assessed using data from days when a human-to-mosquito transmission experiment was done and no asexual or gametocytes were detected in the patients' blood by microscopy, and comparing with measures taken when either or both parasite life stages were present in the blood. Mosquito feeding was more likely to be carried out if gametocytes were present and, therefore, we weighted the analysis by gametocyte status and by stage of infection (grouped into days 1–20, 21–40, 41–60, 61–80, 81–100, 101–200 and >200) so that measures were representative of an average infection.

Other human infectiousness studies. We searched the literature to find as many studies as possible measuring human-to-mosquito transmission from individuals in malaria-endemic areas with neither asexual parasites nor gametocytes detectable by microscopy, using PubMed and modern transmission studies[17,31] as starting points and searching through the relevant literature using bibliographies and a review[8]. We identified three further relevant studies in addition to the malaria therapy data. One of these directly measured submicroscopic parasitaemia using quantitative nucleic acid sequence-based amplification as well as slide positivity[31]. Two further human-to-mosquito transmission experiments measured the infectiousness of slide-negative individuals, but their infection status was not tested by molecular methods[7,30]. We estimated the PCR prevalence in these study populations using the log linear model described in the main text (equation 1, Fig. 1a) and the reported slide prevalence in the study. The prevalence of submicroscopic carriage was calculated as:

$$\text{PCR prevalence} - \text{slide prevalence}$$

We assumed all infections from slide negatives arose from these submicroscopic carriers, using them as the denominator in calculating infectiousness. The contribution of slide-positives or submicroscopic carriers to the infectious reservoir was calculated as:

$$\text{Prevalence} \times \text{proportion mosquitoes infected}$$

The proportion of mosquito infections which would originate from submicroscopic infections was estimated as:

$$\frac{\text{Contribution of submicroscopic carriers to the infectious reservoir}}{\text{Contribution of slide-positive carriers} + \text{contribution of submicroscopic carriers to the infectious reservoir}}$$

Age-prevalence data. We extracted the data from all studies which included children and adults and which gave a breakdown of prevalence by microscopy and PCR for at least three age groups. We fit a linear relationship between microscopy sensitivity and age, using the midpoint of the age group, and tested whether underlying population PCR prevalence was a modifying factor. Here we used log prevalence ratios, as log ORs of microscopy: PCR positivity would decline as PCR prevalence increased, even with constant sensitivity in all settings.

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Acknowledgements

Author contributions

L.C.O. updated the systematic review, did the analysis and drafted the manuscript, T.B. reviewed the studies and collected data, J.T.G. advised and contributed to the analysis, A.L.O. contributed data. All authors contributed to interpretation of data, writing and revising the manuscript, and have seen and approved the final version.

Additional information