Variation in the Prevalence of Submicroscopic Malaria Infections: Historical Transmission Intensity and Age as Key Determinants

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Abstract:

Adoption of molecular techniques to detect *Plasmodium falciparum* infection has revealed large numbers of individuals with low density infections unidentified by traditional microscopy-based approaches, yet able to transmit the parasite to mosquitoes. Although previous studies have found that infections in older, more immune individuals are more likely to be submicroscopic, in lower transmission settings where individuals generally develop less immunity, a larger proportion of infections are also typically submicroscopic. Here we present an updated systematic review to explore this apparent paradox across a range of transmission settings to define when and where submicroscopic infections are likely to be most relevant to malaria control efforts. Our analyses identify the marked influence of age on submicroscopic parasite carriage, with adults (>15 years old) consistently more likely to harbour these infections than children (5-15), in turn more likely than infants (0-5). We also highlight marked geographical variation in the proportion of submicroscopically infected individuals across the globe. This variation can be largely explained by the historical patterns of transmission characterising each area, with the submicroscopic reservoir largest in regions with historically low levels of transmission. These differences in submicroscopic epidemiology across different settings may warrant different approaches to targeting this sub-group if malaria is to be controlled most effectively in the approach to elimination.

Introduction

The ability to accurately detect malaria infection during population surveys in endemic areas is a cornerstone of effective surveillance and control of the parasite. Routinely, this is undertaken using light microscopy of blood films, or increasingly, rapid diagnostic tests (RDTs). Recent years have seen the increased usage of more sensitive molecular methods in research contexts. These techniques (typically PCR-based[1]) have revealed the widespread presence of infections with parasite densities lower than the threshold of detection for routine methods[2–8], challenging traditional views of malaria infection and epidemiology.

Submicroscopic infections are present across a range of different settings and populations[5,9–11]. Although rarely causative of severe, acute symptoms, they have been associated with a number of adverse outcomes during pregnancy[12], as well as mild anaemia[13] and various other symptoms (coughing, vomiting, jaundice etc.) in children under 10 years[14]. In addition to their capacity to cause low-grade disease, these infections may also hold public health relevance due to their contribution to onwards transmission of the parasite. Although typically characterised by lower parasite densities than microscopically detectable infections, individuals with submicroscopic infections frequently harbour gametocytes (the transmissible form of the parasite) and are capable of infecting mosquitoes[15–17]. Moreover, recent work suggests that there is often overlap in the parasite densities (as assessed by quantitative PCR) of individuals that are detectable by microscopy and those who are submicroscopically infected, suggesting that differences in parasite densities between these infections may not always be that significant in practice[17]. Indeed, submicrosopically infected individuals have been shown to contribute to transmission of the parasite across different contexts, including areas of high[18] and low[19] transmission intensity, as well both seasonal[15] and perennial[16] settings (although evidence of their capacity to maintain transmission remains outstanding). Despite this potential relevance to transmission however, our understanding of submicroscopic infections, particularly the

factors influencing the size and extent of the submicroscopic reservoir across infected populations, remains far from complete.

Previous systematic reviews and meta-analyses have found that microscopy misses on average, half of all *P. falciparum* infections compared to PCR-based diagnostic methods in endemic country cross-sectional surveys[20], and that the proportion of infections that are submicroscopic decreases as overall prevalence (reflecting transmission intensity) increases. This is consistent with analyses of the distribution of parasite densities as assessed by quantitative molecular methods, which have also shown that median parasite densities typically increase as transmission intensity increases[17]. These reviews have also demonstrated a clear influence of age on submicroscopic parasite carriage, with adults more likely to harbour submicroscopic infections compared to children[21]. Unresolved questions remain however; whilst this age-related phenomenon has previously been attributed to a greater degree of immunity in adults compared to children, paradoxically, low transmission settings (i.e. those in which the extent of immunity within the population would be expected to be minimal) have been shown to possess the largest proportion of submicroscopic infections[22]. Moreover, previous reviews have also highlighted extensive variation in the size of the submicroscopically infected population across settings which could not be explained by age and transmission intensity alone, suggesting the existence of other important factors that shape and determine the size of the submicroscopic reservoir. Sufficient data to examine to examine very low transmission areas in detail also remained outstanding- it has been hypothesised that the sensitivity of microscopy may increase due to the near-absence of immunity in these settings but exploration of this hypothesis in previous reviews had not been possible. Resolving these gaps in our understanding of submicroscopic epidemiology is key to understanding when and where submicroscopic infection is likely to occur, and how the size of the submicroscopic reservoir is likely to change as areas approach elimination.

Addressing these questions has material consequences for the future of malaria control. Despite mixed reports surrounding more recent progress[23], transmission is still declining in many endemic

countries and the World Health Organisation has identified 21 countries that have the potential to eliminate by 2020 (http://www.who.int/malaria/areas/elimination/e2020/en/). Low transmission settings such as these can possess high proportions of submicroscopically infected individuals[22,24]. For example, the existence of a significant submicroscopic infection prevalence in an otherwise asymptomatic, microscopically negative population in Ethiopia[25] highlights that malaria infections can persist in populations entirely absent of clinical indicators or detectable using typically deployed diagnostics. Understanding the prevalence, detectability and infectiousness of low density infections in these settings will be essential for planning for elimination: do they need to be specifically targeted to interrupt transmission; are more sensitive diagnostics required to detect these infections; and how sensitive are these answers to the rate at which a country approaches elimination?

Here we update previous reviews on submicroscopic malaria infection prevalence[20,22], leveraging the large increase over the past 5 years in the usage of molecular methods to enable exploration of new determinants of submicroscopic infection prevalence. We explore the influence of historical patterns of transmission and geographical location on the extent and size of the submicroscopic reservoir, as well as the role of age at a finer resolution than has previously been possible. The results of these analyses are then combined with estimates from the literature describing the infectivity of submicroscopic individuals to mosquitoes in order to estimate the contribution of this subgroup to malaria transmission across a range of different transmission settings.

Results

Microscopic detectability of infections across the endemicity spectrum. Survey data detailing *P. falciparum* prevalence as measured by both microscopy and PCR in the same individuals was compiled and collated, through updating two previous reviews last completed in 2011[20,22]. 57 surveys (representing a total of 177 microscopy and PCR prevalence survey pairs) met our inclusion criteria, namely that they took place in an endemic population within a defined geographic area, and that participants were not selected according to malaria symptoms or test results (see also Methods).

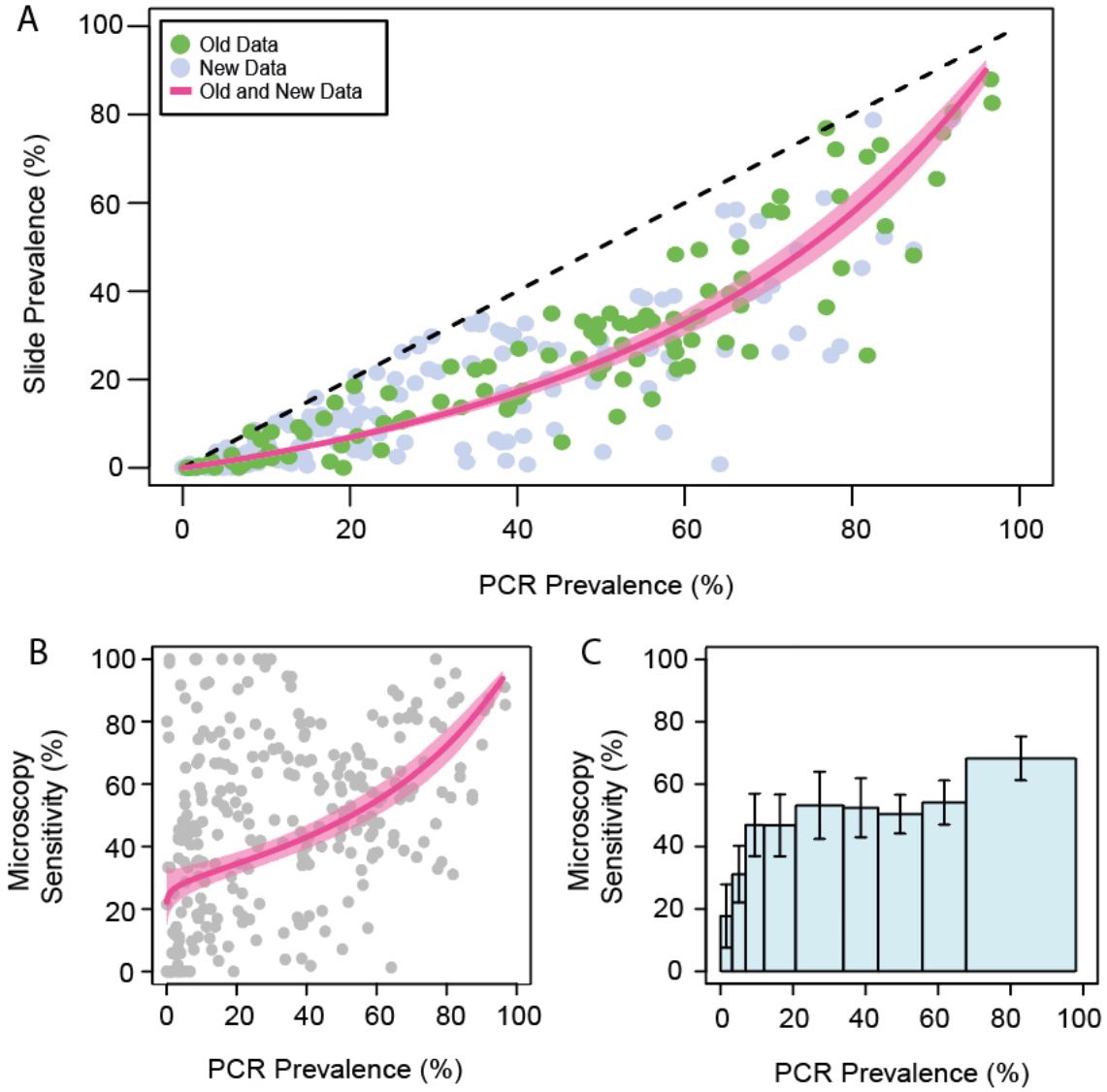

Figure 1: Prevalence of infection by PCR versus microscopy in 282 prevalence survey pairs and model fits. Bayesian Markov chain Monte Carlo methods were used to fit a linear relationship between PCR prevalence and microscopy prevalence on the log odds scale. **(A)** Microscopy and PCR Prevalence data (n = 282) from surveys identified in this systematic review (light purple points, n = 177) and in previous reviews (green points, n = 105), with the fitted model relationship (pink line) and the 95% credible interval of the mean (pale pink shaded area). **(B)** The sensitivity of microscopy (defined here as the proportion of PCR positive individuals also detectable by microscopy) according to underlying PCR prevalence for each of the 282 survey microscopy-PCR pairs (grey points) used to fit the full model. The estimated average sensitivity (pink line) and 95% credible interval of the mean (pale pink shaded area) also shown. **(C)** Empirically estimated microscopy sensitivity. Survey pairs were categorised into 9 categories of equal size and mean microscopy sensitivity empirically estimated for each category. Error bars represent the binomial 95% confidence interval.

Submicroscopic infections were defined as those where the infection was detectable by PCR but not by microscopy. Across the 103 surveys (representing 282 microscopy and PCR prevalence survey pairs) included in our analyses (including both references identified in this systematic review and those from the previous reviews), microscopy detected on average 46.8% of all PCR detectable infections (43.5% - 50.2%, 95% confidence interval (CI)), although this varied widely across surveys, as with previous analyses[20,22] **(Figure 1A)**. There was no detectable difference in the observed relationship between PCR and microscopy prevalence when the model was fitted separately to data collated in previous reviews and the data newly extracted here **(see Supplementary Figure 1)**. The overall fitted relationship between PCR prevalence and microscopy prevalence for the combined dataset was:

Log odds PCR prevalence = 1.084 + 0.947 * Log odds microscopy prevalence

where log odds = ln(prevalence/(1-prevalence) and prevalence = exp(log odds)/(1+exp(log odds)). The average proportion of submicroscopically infected individuals as a proportion of the total infected population decreases as malaria transmission increases, from between 70% and 80% in the areas of lowest PCR prevalence to only 10-20% in the highest prevalence areas. There is, however, large variation in the proportion of submicroscopic infections between surveys carried out in regions with similar PCR prevalence **(Figure 1B).** The new data identified in this review gave a much richer picture of extremely low transmission settings. We observed no upward trend in the mean sensitivity of microscopy at very low transmission settings (0.04 to 3.23% PCR prevalence, where population immunity might be expected to be lowest) compared to regions of moderate transmission intensity **(Figure 1C)**. Based on these results, the following sections explore the influence of a number of factors that might explain both the general trend as well as the marked variability observed.

The influence of age on submicroscopic parasite carriage. Both increasing age (independent of exposure) and increased immunity (due to previous exposure, which also increases with age) have been linked to lower parasite densities[26,27]. Previous reviews of submicroscopic epidemiology have confirmed this, although data limitations have previously restricted analyses to only two age

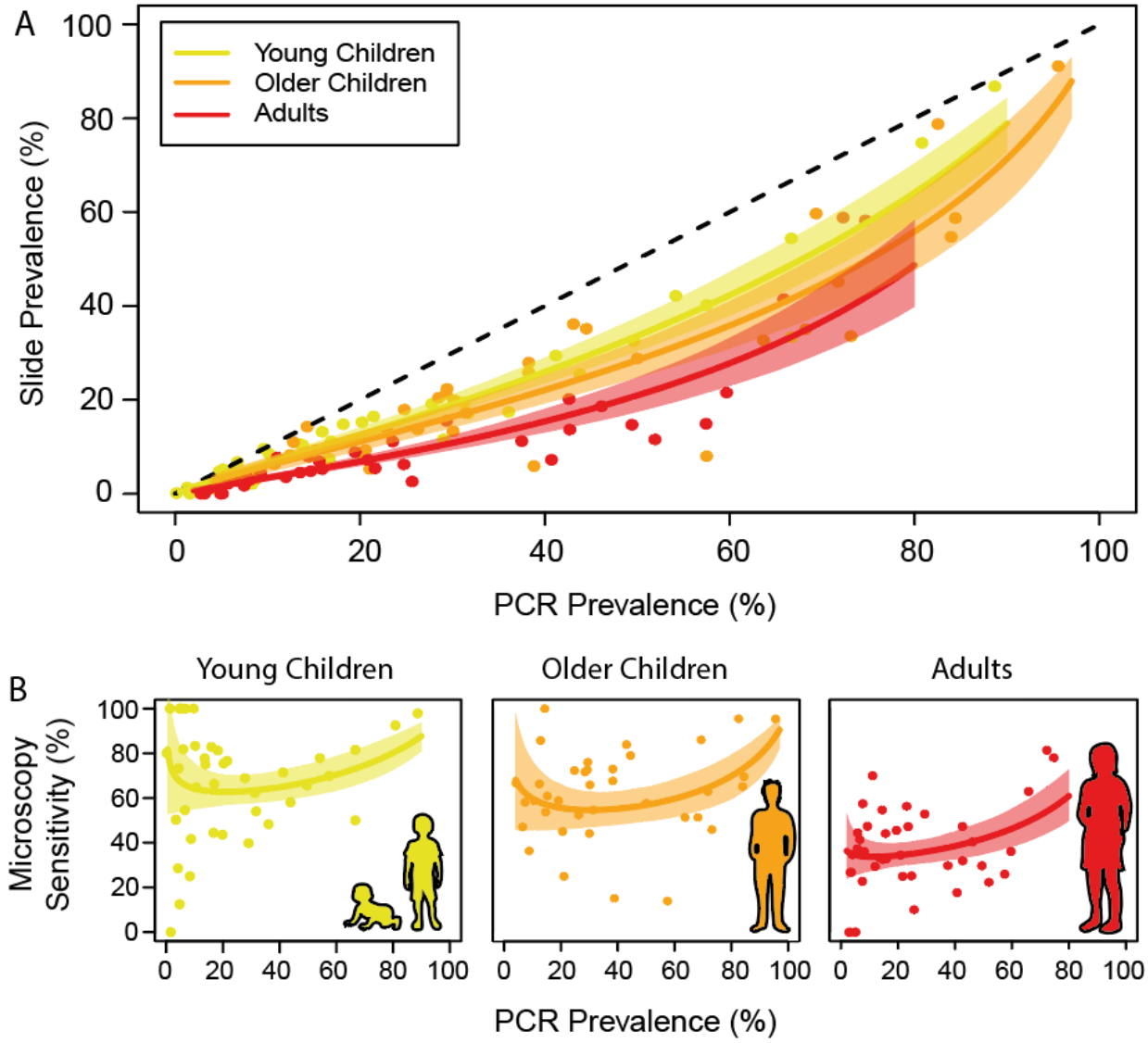

Figure 2: The influence of age on microscopy sensitivity and submicroscopic parasite carriage. Separate models were fitted where age disaggregated data were available to assess whether the prevalence of infection by microscopy compared to PCR varied with age. **(A)** Age disaggregated prevalence survey data for young children (0-5 years old, yellow points, **n = 40)** older children (5-15 years old, orange points, **n = 37)** and adults (red points, **n = 41)** with the fitted model relationship (coloured line) and 95% credible interval for each (shaded area). **(B)** The sensitivity of microscopy compared to PCR in surveys where age-disaggregated data (dots) were available by age group, showing the fitted model relationship (coloured lines) and the 95% credible interval (shaded areas).

categories (≤ 16 and > 16 years old), with a limited number of datapoints in each (n = 28 and n = 13 respectively)[22]. The increased quantity of data in the updated review allowed us to define 3 age-based categories for analysis: infants (0-5 years old), older children (5-15 years old) and adults (>15 years old), yielding 34, 36 and 42 datapoints which were used to explore the influence of age on the prevalence of submicroscopic infections. Analysis of these results in aggregate revealed that average microscopy sensitivity varied significantly between age groups (ANOVA, $p < 0.001$, df = 2), with sensitivity of microscopy in adults found to be consistently and significantly lower than that observed both for young children (Tukey's HSD, $p < 0.001$) and older children (Tukey's HSD, $p < 0.001$). Mean sensitivity was lower in older children compared to young children, although this difference was not significant (Tukey's HSD, $p = 0.61$).

We next explored whether these differences were observed consistently across the range of transmission settings (characterised by population PCR prevalence as a measure of transmission intensity) (Figure 2). Fitting the regression model separately for each age group highlighted that the increased sensitivity observed in infants and older children compared to adults was less pronounced in higher transmission settings. These results suggest genuine differences in submicroscopic epidemiology both between age groups and across transmission settings, a phenomenon likely representing a complex interplay of exposure, immunity and other, poorly resolved age-related processes.

Global variation in microscopy sensitivity and the size of the submicroscopic reservoir. Although evidence suggests that the burden of submicroscopic infections is highly heterogeneous across different locations[9,28], it remained unclear whether this represented systematic variation according to geography. Grouping surveys into 4 global regions (West Africa, East Africa, South America, and Asia and Oceania) revealed significant geographical variation in sensitivity (ANOVA, $p < 0.001$, df = 3), with microscopy sensitivity consistently lower in South American surveys compared to other regions (Tukey's HSD, $p < 0.001$ for all 3 pairwise comparisons with other global regions).

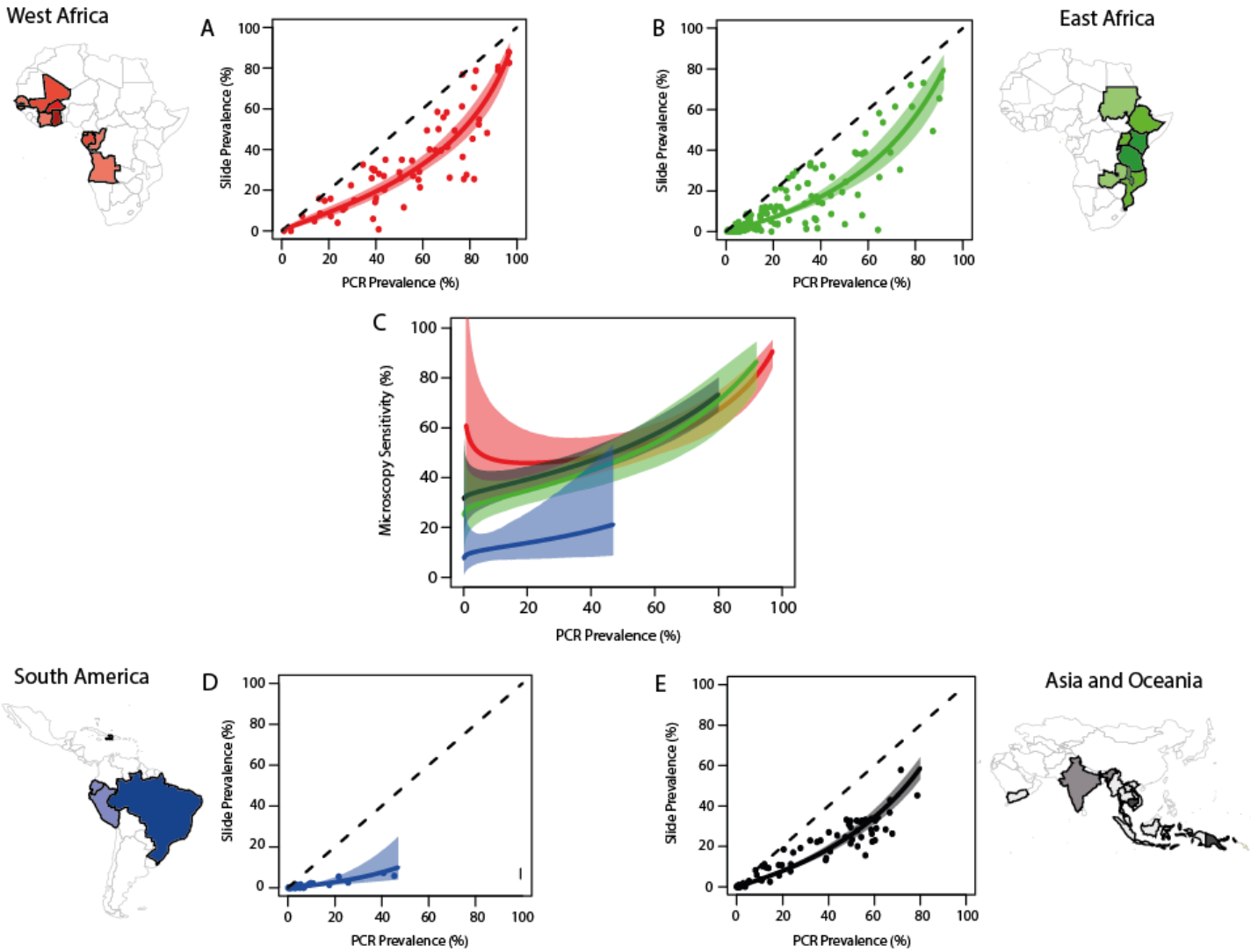

Figure 3: Global variation in the sensitivity of microscopy. Microscopy and PCR prevalence in included surveys (points), the model fitted relationship (coloured line) and 95% credible interval (shaded area) for **(A)** West Africa **(n = 63)**, **(B)** East Africa **(n = 121)**, **(D)** South America **(n = 27)** and **(E)** Asia and Oceania **(n = 71)**. **(C)** The average sensitivity of microscopy compared to PCR based on the fitted model for each of the 4 regions (coloured) line and 95% credible interval (shaded area). Intensity of colour for each country on each regional map indicates the comparative number of studies for each country, compared to other countries in that same region.

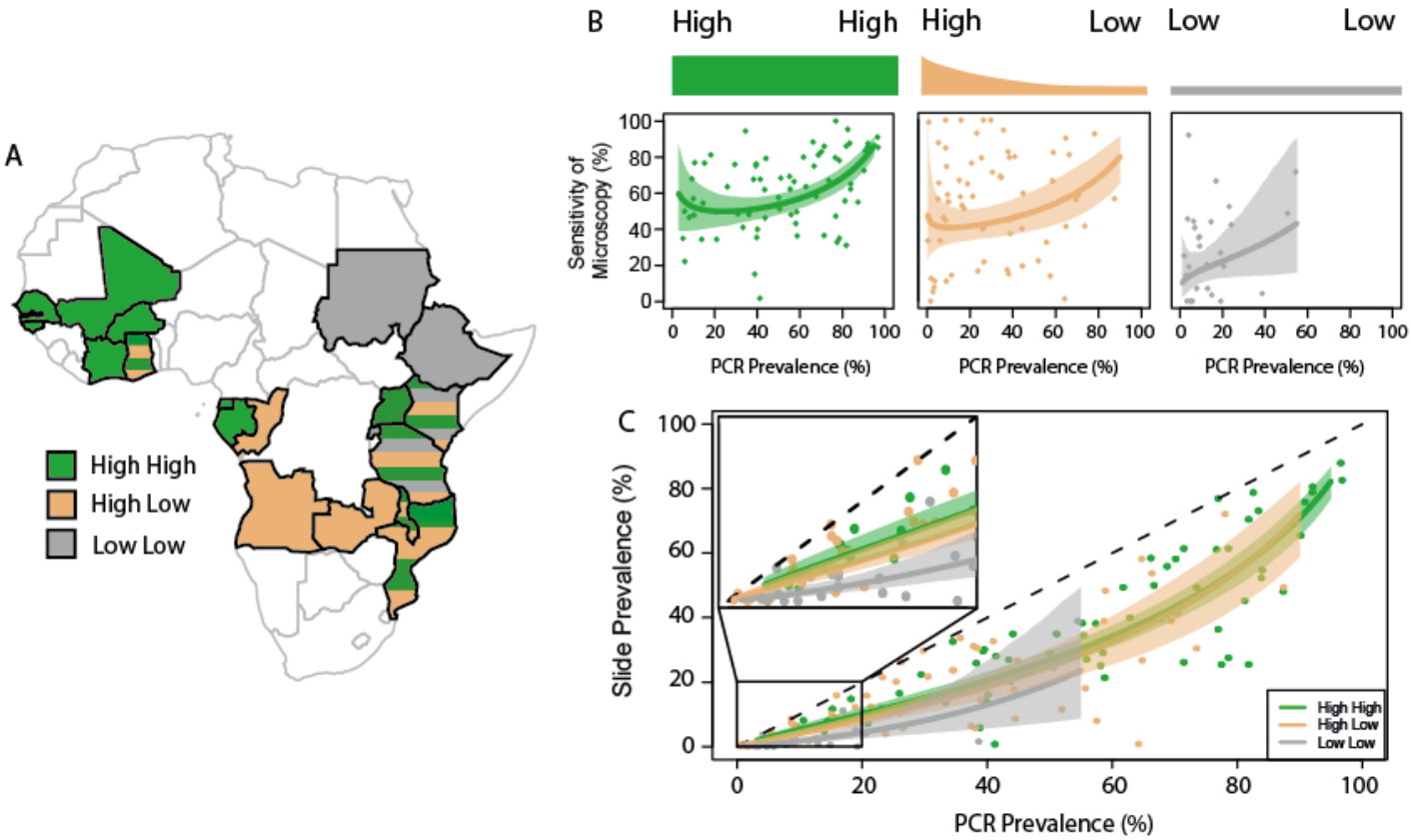

Figure 4: The effect of historical and current transmission intensity on the sensitivity of microscopy. Historical estimates of malaria prevalence[29] were used to assign surveys in Africa to one of three transmission intensity archetypes, and then separate models fitted to each to explore whether variation in the present and historical levels of transmission in a region might influence microscopy sensitivity. **(A)** Map detailing the African countries for which prevalence surveys were identified, as well as the transmission archetype surveys in these countries were assigned to. Multiple colours mean that the country contained >1 different transmission archetype in different administrative units. **(B)** Sensitivity of microscopy compared to PCR for surveys in each transmission archetype (dots, n = 71, 65 and 28 for historically and currently high, historically high, currently low and historically and current low settings respectively), as well as the average sensitivity (coloured line) and 95% credible interval (shaded area) generated from the model fitting. **(C)** The model fitted relationship (coloured lines) and 95% credible interval (shaded areas) for each of the transmission archetypes, with a particular focus on low prevalence (<20%) settings (inset box).

However, these analyses of mean microscopy sensitivity were confounded by the fact that average transmission intensity (as measured by PCR prevalence) also systematically differed across all 4 locations. To account for this, we fitted separate regression models to data from each of the 4 global regions **(Figures 3A, 3B, 3D and 3E)**. In doing so, we controlled for the variable transmission intensities each survey was carried out in. These results revealed that in surveys from South America, microscopy sensitivity was still lower than would be expected based on their respective transmission intensities alone **(Figure 3C).** This points to the role of additional factors in shaping the relationship between transmission and microscopy sensitivity other than just endemicity levels.

Exploring the influence of historical and current transmission intensity. A clear feature of the majority of the South American surveys was that they were carried out in areas of historically low transmission. However, it remained unclear whether the results observed for South America reflected genuine geographical variation or were instead a product of past patterns of transmission in the regions where the surveys had been undertaken. We therefore investigated whether a high proportion of submicroscopic infections might be observed in other areas around the world that had experienced similar historically low patterns of transmission. Surveys in our review from Africa (a continent for which estimates of malaria prevalence are available at a high spatio-temporal resolution) were located to administrative unit 1 (subnational) areas, and estimates of malaria prevalence extracted from the Malaria Atlas Project maps[29]. Surveys were then assigned to one of three categories dependent on current and historical transmission- Historical & Currently High (Prevalence >15% both prior to 2000 and at the time of survey), Historically & Currently Low (Prevalence <15% both prior to 2000 and at the time of survey), and Historically High, Currently Low (Prevalence >15% prior to 2000, <15% at the time of survey). If transmission history rather than geographical location was driving the result we observed for South America, we would expect African surveys in locations where transmission is and has been low for a long time to show similarly low microscopy sensitivities.

Initial analyses revealed that African surveys in areas with both historically and currently low transmission (Sudan, Ethiopia and parts of Kenya and Tanzania) **(Figure 4A)** had lower microscopy sensitivities on average than areas which currently have similarly low transmission but are marked by historically high transmission and those where transmission intensity remains high (ANOVA, $p < 0.001$, df = 2). This result was also observed following fitting of separate regression models to the stratified data **(Figures 4B and 4C)**. By contrast, sensitivity was broadly equivalent across areas of historically high transmission, irrespective of whether transmission remained currently high or had recently declined to low levels. Observation of this pattern across African surveys therefore suggests that the low sensitivity observed in South America is reflective of the common history of low transmission characterising these locations.

Contribution of the submicroscopic reservoir to onwards transmission across different transmission archetypes. Submicroscopic infections can contribute substantially to malaria transmission across a range of different settings[19,30]. We therefore utilised model predictions of the proportion of submicroscopic infections across different transmission archetypes (characterised by their historical and current levels of transmission) to estimate how the contribution of these infections to onwards transmission might vary across different settings **(Figures 5A, 5B and 5C)**. Due to uncertainty in the relative infectivity of submicroscopic infections compared to microscopic infections, we explored a number of different, plausible scenarios of comparative infectivity (2x, 5x and 20x, see Methods). In transmission settings characterised by historically low levels of transmission, and even under the most conservative of estimates of submicroscopic infectivity (that assumes microscopically detectable infections are 20x as infectious as submicroscopic ones), submicroscopically infected individuals could account for 24.2% of onwards transmission **(Figure 5C)**. This figure is substantially higher under less conservative assumptions. Using the current best estimate of comparative infectivity from a recent metanalysis which indicated that on average, microscopically detectable infections are 2.78x more infectious compared to submicroscopic infections[17], estimates of contribution to onwards transmission rise to 75%. By contrast, our results suggest the contribution of the submicroscopic

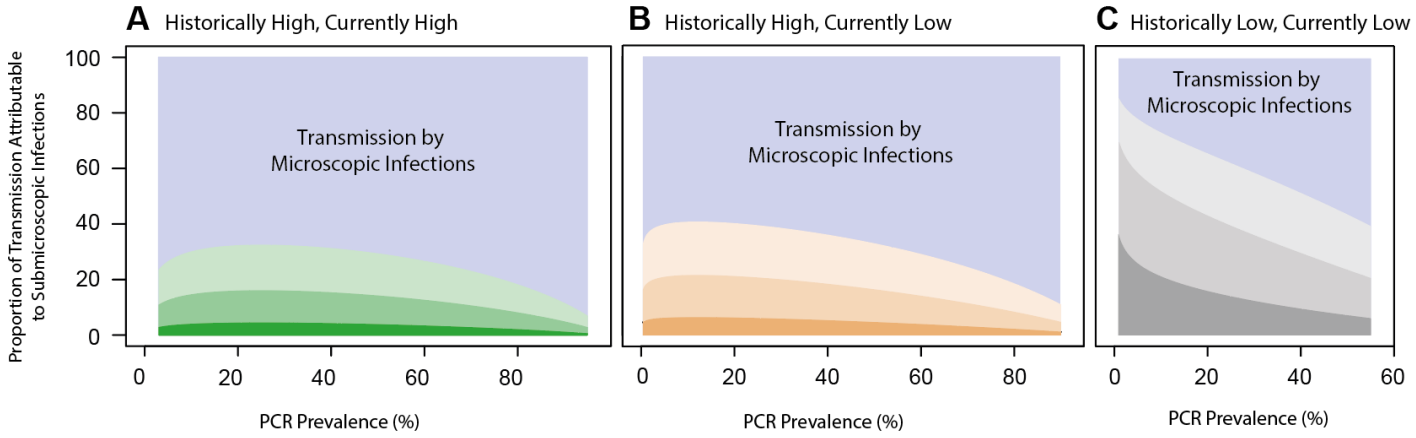

Figure 5: The potential contribution of submicroscopic infections to onwards transmission across different settings. The modelled relationship between PCR and microscopy prevalence previously derived for each of the transmission archetypes was used to estimate the predicted proportion of submicroscopic infections in each of these types of setting, across the range of PCR prevalences empirically observed in the gathered surveys. Given this predicted proportion, the potential contribution of the submicroscopic reservoir to onwards transmission was then explored **(A, B and C)**, using a range of different assumptions (supported by existing literature) surrounding the comparative infectivity of microscopic and submicroscopic infections- that microscopic infections are either 2x, 5x or 20x more infectious than submicroscopic infections. In each case, the purple shaded area represents the proportion of transmission attributable to microscopically detectable infections, and the coloured shaded area the contribution of submicroscopic infections for 1 of the 3 assumed comparative infectivities- darkest shade is 20x (i.e. that microscopically detectable infections are 20x more infectious than submicroscopic infections), intermediate shade is 5x, and lightest shade is 2x.

reservoir to onwards transmission is less important in low transmission settings where transmission has only recently declined **(Figure 5B)**, although their contribution is not irrelevant.

Discussion

Significant debate still surrounds the importance of the submicroscopic reservoir to transmission of the parasite and malaria control efforts, particularly regarding how this importance varies between settings[31]. Disaggregating the now larger quantity of available data (representing 282 prevalence survey pairs from 38 countries) has yielded insight into the complex relationships underlying the global pattern of submicroscopic occurrence, facilitating a more refined evaluation of where submicroscopic infections are likely to be most numerous and who is most likely to harbour them. In particular, we show that the transmission gradient in microscopy sensitivity observed across all settings is likely driven primarily by differences in the historical patterns of transmission and the age structure of the infected population. Moreover, whilst previous work has generally noted the potential relevance of submicroscopic infections in low transmission settings[32], our results suggest that their relevance is likely to be highly context dependent, potentially warranting different approaches to their control depending on the setting.

It is widely understood that malaria prevalence across different settings varies with age[33], a feature attributed to the development of immunity after exposure, as well as age-related effects apparently independent of exposure[26,34]. As a result, both increasing age and increased immunity have been linked with lower parasite densities[26,27] and our analyses confirmed a significantly higher proportion of submicroscopically infected individuals in surveys of adults compared with children. Whilst there is significant uncertainty in the results associated with surveys in very low prevalence ranges (due to the small number of infected individuals in each study), the current analysis found systematically lower microscopy sensitivities in regions characterised by a history of low transmission, a result that was observed across both South America and Africa. This phenomenon is unlikely to be a product of lower

expertise of local microscopists in these settings; a recent review of the literature has found that whilst microscopy quality varies across settings, it does not do so systematically with transmission intensity[17]. An important additional caveat to these findings is that there were insufficient data to examine the role of age and transmission history simultaneously. The average age of infection is typically higher in low transmission settings[33,35]; and therefore , a greater proportion of infected individuals in all-age surveys will be adults. Our results surrounding past transmission history might then be confounded by differences in the average age of infection (and hence the underlying age structure of the infected population) across these different settings. However, the age distribution of malaria infection typically adapts rapidly to reflect changes in transmission, and therefore this confounding is unlikely to explain our finding of a lower proportion of submicroscopic infections in areas of current low transmission but where transmission has historically been high. For example, it was shown that in an area of south-western Senegal experiencing a 30-fold drop in malaria incidence between 1996 and 2010, there was a shift in the age distribution of cases from predominantly under-5s (34% of cases in 1996) to a distribution that was near uniform across the population[36]. Similarly, rapid declines in malaria between 2003 and 2007 were shown to be accompanied by an increase in the mean age of paediatric malaria admissions from 3.9 years to 5.6 years[37]. These primarily clinical results also extend to the prevalence of malaria infection (as assessed by microscopy) in wider populations and communities [35]. Together, these observations suggest that the difference in infected population age structure between surveys from historically and currently low settings and those from settings where transmission has only recently declined may not be large. The observed disparity in microscopy sensitivity between settings with different historical patterns of transmission therefore supports an effect of transmission history on the submicroscopic reservoir which is distinct from the age structure of the infected population. Furthermore, the observation of a greater submicroscopic reservoir in historically low transmission across both Africa and South America supports the contention that these results are a reflection of the common history of low transmission these locations share.

Although several hypotheses could account for these results, one possible explanation might be differences in the extent of parasite genetic diversity between settings. Areas of high prevalence typically possess markedly higher genetic diversity compared to areas where transmission is and has been low for a significant period of time[38]. Experimental infection studies have demonstrated that immunity arising from infection with a single strain appears to be stronger[39,40] (and leads to lower parasite densities[41]) against that same strain than other strains (homologous versus heterologous immunity), a phenomenon observed across non-human experimental systems[42], experimental human infection studies[43] and various vaccine-related trials[44]. Given this, in historically low transmission settings, repeated exposure to a limited number of strains might lead to rapid development of protective immunity against those strains. Individuals in these settings would then be expected to have, on average, a higher degree of immunity to potential infections- by extension, a higher proportion of the infected population would be expected to submicroscopic **(Figure 6A)**. By contrast, in historically and currently high transmission settings, higher circulating parasite genetic diversity would mean that individuals are more frequently infected with strains they have not previously encountered **(Figure 6B)**. Moreover, given the frequently observed lag between reductions in transmission and reductions in circulating genetic diversity of the parasite[45–49] (a result also supported by work modelling parasite genetic diversity under control efforts[50]), parasite genetic diversity in historically high regions where transmission has recently declined might then be expected to be most similar (although not identical) to historically and currently high transmission regions **(Figure 6C)**. These two transmission archetypes would then be expected to show similar patterns of microscopy sensitivity, a feature we observe in the results presented here.

Considerations of circulating genetic diversity might go some way to explain recent results within countries that are discordant with the global pattern linking PCR prevalence and microscopy sensitivity observed when considering all survey pairs together. Recent characterisation of submicroscopic malaria carriage at three Ugandan sites with varied transmission intensity revealed little change in the extent and size of the submicroscopic reservoir across the transmission gradient at this sub-national

level[10]. Similarly, a micro-epidemiological exploration of submicroscopic parasite carriage in a rural area of north-west Tanzania found the odds of an infection being submicroscopic to actually increase with household exposure[51]. Under an assumption of spatial circulation of strains between subnational localities, mixing of parasites between sites of varying transmission intensity but spatial proximity would mean they possess similar extents of parasite genetic diversity[52], and by extension, submicroscopic burdens. This would be despite marked differences in levels of local transmission.

Important to note however is that a number of other hypotheses could also explain the results we observe here. Substantial variation exists in the asexual blood stage multiplication rate of *Plasmodium falciparum*[53], with recent work linking this variation in parasite biomass during infection to genetic variation in the immune dominant antigen *Pf*EMP1[54]. It is possible then that systematic differences across settings in the virulence or growth capacity of the parasite could account for the observed results. It is also not possible to definitively preclude systematic variation in the quality of PCR techniques across surveys as a factor. This is perhaps unlikely however given previous reviews of submicroscopic epidemiology have found no significant association between microscopy sensitivity and variables associated with PCR methodological quality (including the volume of blood used for DNA extraction, the PCR protocol and the quantity of extracted DNA used in the PCR)[22] and that no significant differences in detection accuracy were observed during a recent review of the most commonly used PCR-based assays[55]. Additionally, the relationship we observed between PCR and microscopy prevalence was near identical when the model was fitted only to data from previous reviews or the data newly collated here (Supplementary Figure 1), suggesting that systematic or temporal variation in PCR quality are unlikely to be underpinning the results presented here.

More broadly, that there are a number of additional factors associated with submicroscopic carriage not analysed here. These range from host genetics, such as the various haemoglobinopathies and human genetic traits that have been linked to lower average parasite densities[34,56] to seasonal effects, evidenced by the fact that parasite densities slightly rise during the rainy season even when

prevalence does not change significantly (possibly due to an increased occurrence of superinfection during these periods of high transmission intensity)[17,57]. Outside of seasonal settings, superinfection is still an important consideration, given it can increase parasite densities[58] and would be expected to be more common in higher transmission settings. Superinfection was previously hypothesised to be a major determining factor in the lower proportion of submicroscopic infections in higher transmission areas[21], but this theory is unable to explain the large differences we now observe in areas with different transmission histories but similar current levels of transmission.

Our work exploring contribution to onwards transmission suggests that the contribution of submicroscopic infections to onwards transmission is likely to be highly variable across settings, ranging from only 1% (in historically and currently high transmission settings, assuming microscopically detectable infections are 20x more infectious than submicroscopic infections), to as high as 80% (in historically and currently low transmission settings and assuming an infectivity ratio of 2x). This suggests that the relevance of submicrosopically infected individuals to malaria control is also likely to be highly context dependent. It is important to note however that this analysis is based on the detection of asexual parasites and does not provide direct insight into the sexual stages of necessary for onwards transmission. Although there is a relationship between microscopically detectable asexual infection and gametocyte presence[59], the relationship between asexual parasite and gametocyte density is less clear. Moreover, the distributions of parasite densities in the submicroscopic range differs from setting to setting (by as much as several orders of magnitude[17]), an important consideration that will also shape their contribution to onwards transmission and that is not considered here. It is therefore not necessarily the case that the proportion of submicroscopic infections relates linearly and directly to contribution of this infected sub-group to onwards transmission across different settings. For example, a recent membrane feeding study conducted in Burkina Faso and Kenya (high transmission settings) using both microscopically and submicroscopically infected individuals found that 45-75% of all mosquito infections that arose from feeding were derived from submicroscopic infections[30]. By contrast, only 4% of infections arose from

submicroscopic individuals in a recent membrane feeding study carried out in Cambodia (a low transmission setting)[60]. These findings contrast with those predicted by our results and underscore the need for further work to better resolve the relationship between submicroscopic parasite carriage, gametocyte densities, and individual infectivity to mosquitoes.

Although progress has stalled more recently, substantial enthusiasm still surrounds attempts to eliminate malaria[61]. Indeed, 21 countries have been identified by the WHO as having the potential to achieve zero indigenous cases of malaria by 2020 (the E-2020 initiative, https://www.who.int/malaria/areas/elimination/e2020/en/). Despite this however, and despite their potential relevance to transmission of the parasite, our understanding of submicroscopic infections in these settings remains far from complete. Do they represent a substantial source of transmission and threat to future progress in places where the microscopically detectable population may only represent the tip of the (transmission) "iceberg"[62,63]? Or do they simply reflect the remnants of infections capable of transmission, more akin to dying embers (and thus less of a concern)? Our work highlights the likely absence of a one-size-fits-all solution for malaria control in settings approaching elimination and instead suggests that important differences in submicroscopic epidemiology across settings might well warrant different approaches and considerations surrounding this infected sub-group if malaria is to be controlled most effectively in the approach to elimination.

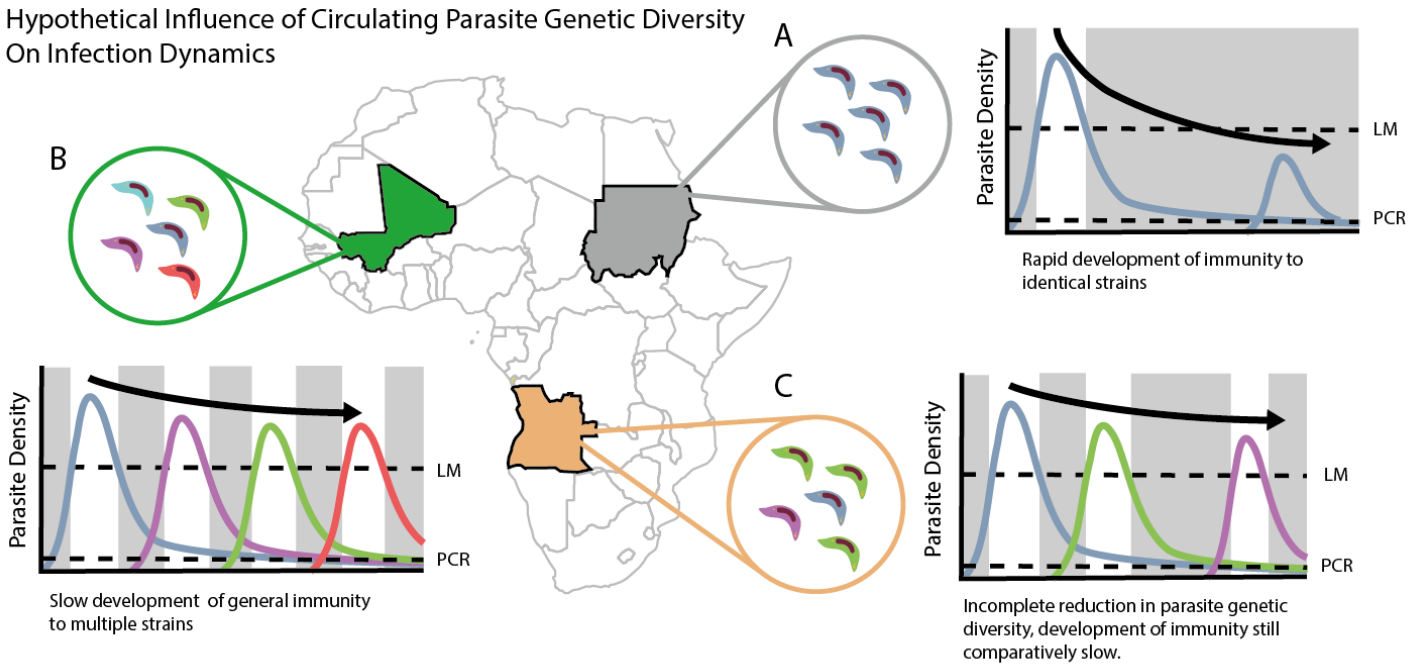

Figure 6: Hypothesised infection dynamics within individuals across settings with differing histories of transmission. Schematic illustrating hypothesised dynamics of parasitaemia in an individual across different settings: either where transmission has been **(A)** Historically and Currently Low, **(B)** Historically High and Currently High, or where it has been **(C)** Historically High but has recently declined and is Currently Low. Circles pointing towards representative countries qualitatively illustrate the extent of genetic diversity that would be expected to be found in each setting- different coloured parasites represent genetically distinct *Plasmodium falciparum* strains. The graphs represent the within-host infection dynamics and measure parasite density over time. Different coloured lines represent infections with genetically distinct strains, with periods where the infection is submicroscopic (grey areas) and periods where the infection is detectable by microscopy (white areas) detailed. Black dotted lines indicate the detection limits of microscopy (LM) and PCR. Black solid arrows are used to illustrate the decline in parasite density over time during subsequent infections.

Methods

Systematic Review Update and Data Extraction

Malaria prevalence data where both microscopy and PCR based methods had been used to determine infection status was compiled, updating a previous systematic review published in 2012[22] using the same criteria. Briefly, searches were carried out using PubMed and Web of Science databases and the search terms ("PCR" OR "Polymerase Chain Reaction") AND "falciparum". Records published between January 2010 and July 2017 were searched for, and together yielded 2136 records. Studies reporting data on asexual *P. falciparum* prevalence by microscopy and PCR in the same population were included. Surveys of pregnant women were excluded, as were those where study participants had been chosen on the basis of presented symptoms, those not involving a population from a defined geographical area, as well as where individuals had recently received treatment as part of a study (e.g. mass drug administration campaigns).

After screening titles and abstracts, 231 references were retained for full text evaluation, of which 55 were included. A further two relevant references, unpublished at the time of screening but since published, were also included[19,30], as were additional data collected through contacting authors of included references. Overall this yielded a total of 177 new microscopy and PCR prevalence survey pairs. Together with the previous systematic review results, this gave a total of 282 prevalence surveys. Submicroscopic infections were defined as those where the infection was detectable by PCR but not by microscopy. The specificity of microscopy compared to PCR has been shown in a previous review to be high (98.4% on average[20]) and so we assume in our analyses that any microscopy-positive results are also PCR positive, in-keeping with previous reviews[21]. There were a small number of instances where the number of microscopically detected infections was higher than those identified by PCR (n = 10)- the sensitivity in these cases was adjusted to 1 and these adjusted values used in the presented analyses. Additional analyses were conducted with and without these results included, both

when adjusted and when not adjusted and it was confirmed that their inclusion, adjusted in this way, did not qualitatively alter the results produced.

Historical and Current Transmission Intensity Categorisation

Surveys conducted in African settings were geolocated, and then prevalence estimates (aggregated to the administrative unit 1 (subnational areas) level) from the Malaria Atlas Project[64] used to characterise the current and historical transmission intensity of the regions where the surveys had been carried out. We assigned each study to one of the following categories:

- **Historically High, Currently High:** areas that have historically (defined as 15 years previous to the date of the survey) been characterised by high transmission intensity (>15% slide prevalence in 2-10 year olds (*Pf*PR_{2-10})) and which remain so at the time of the survey (n = 71).
- **Historically High, Currently Low:** Areas that had historically high transmission intensity but which have declined in recent years to low levels (<15% *Pf*PR_{2-10}) (n = 65).
- **Historically Low, Currently Low:** Areas characterised by both historical and current levels of low transmission (<15% *Pf*PR_{2-10}) (n = 28).

Where MAP estimates were unavailable (for dates earlier than 2000), it was assumed that the year 2000 was reflective of the historical transmission intensity in the area given the relative lack of scale up of malaria control interventions in most areas before that date, and the only recent substantial increase in international financing for malaria control (an approximately twentyfold increase between 2000 and 2015)[65].

Statistical Analysis and Bayesian Linear Regression

As in previous reviews[21], the data were analysed using the methodology detailed in Sharp et al[66]. A linear relationship was fitted to the log odds of PCR and microscopy prevalence:

$$\Omega_{\mathrm{Mi}} = \Omega_{\mathrm{Pi}} + \delta_{\mathrm{i}} \qquad (1)$$

$$\delta_{\mathrm{i}} = \delta'_{\mathrm{i}} + \beta_0 \Omega_{\mathrm{Pi}} \qquad (2)$$

where Ω_{Mi} is the log odds of microscopy prevalence in trial *i*, Ω_{Pi} is the log odds of PCR prevalence and δ_{i} is the log odds ratio (OR) of microscopy to PCR prevalence. Formulation in this way allows the log OR of Microscopy: PCR prevalence (δ_{i}) to vary between surveys, with β_0 representing a regression coefficient specifying how the log OR changes with log odds PCR prevalence. Both the log OR and log odds of PCR prevalence were assumed to be normally distributed.

The above model was fitted using a Bayesian Markov Chain Monte Carlo (MCMC) based framework, implemented in the statistical software package JAGS[67]. The observed prevalence values were assumed to be drawn from a binomial distribution with the sample size of the survey as the number of trials (people tested), and the prevalence as the probability of success in any given trial. Uninformative prior distributions were assigned to all parameters in the model. The model was initially fitted to all the collated data and then subsequently fitted to various subsets of the data (stratified by geographical location, transmission setting etc.) to assess the role of different factors on the extent of the submicroscopic reservoir.

Calculating Microscopy Sensitivity and Submicroscopic Contributions to Onwards Transmission

The relationship between PCR and microscopy prevalence calculated using the above method can then be used to estimate microscopy sensitivity. Specifically, estimates of β_0 and δ_{i}' were used to calculate the log odds of microscopy prevalence for a given log odds of PCR prevalence. These estimates were then transformed to the linear scale, where the estimate of the prevalence by microscopy was then used to calculate microscopy sensitivity:

$$Sensitivity = \frac{Prevalence\ by\ Microscopy}{Prevalence\ by\ PCR}$$

Given that the sensitivity represents the proportion of total infections detected by microscopy, $1 - Sensitivity$ provides an estimate of the proportion of infections that are submicroscopic.

Using this estimate of the size of the submicroscopic reservoir, we then estimated the size of the contribution of submicroscopic infections to onwards transmission of malaria. Although estimates of the comparative infectivity of microscopic infections compared to submicroscopic infections are available in the literature, such estimates are highly variable across different settings. These range from estimating that microscopic infections are twice as infectious as submicroscopic ones[68] to as high a 20 fold difference[60]. To account for this, we explored three scenarios representing different comparative infectivities, defined as:

$$Infectivity\ Ratio = \frac{Infectivity\ of\ Microscopy_{+ve}\ \ Infections}{Infectivity\ of\ Microscopy_{-ve}\ Infections}$$

where $Microscopy_{-ve}$ is the proportion of infections that are submicroscopic, $Microscopy_{+ve}$ is the proportion of infections that are detectable by microscopy, and therefore $Infectivity\ Ratio$ is the comparative infectivity of microscopically detectable infections compared to submicroscopic ones. Specifically, instances where microscopic infections were 2 times, 5 times and 20 times more infectious to mosquitoes than submicroscopic infections were used (corresponding to an Infectivity Ratio of 2, 5 and 20 respectively). Comparative contributions to transmission were calculated as follows:

$$\frac{Microscopy_{-ve}}{(Microscopy_{+ve}\ x\ Infectivity\ Ratio) + Microscopy_{-ve}}$$

The denominator of this equation therefore reflects the total onwards transmission occurring within the population, and the numerator the prevalence of submicroscopically infected individuals- it therefore yields an estimate of the proportional contribution of submicroscopic infections to onwards transmission. These analyses include an implicit assumption that submicroscopic and microscopically infected populations do not differ in other key factors likely to also influence transmission (e.g. age).

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