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Why do we need to know more about mixed *Plasmodium* species infections in humans?

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Four *Plasmodium* species cause malaria in humans. Most malaria-endemic regions feature mixed infections involving two or more of these species. Factors contributing to heterogeneous parasite species and disease distribution include differences in genetic polymorphisms underlying parasite drug resistance and host susceptibility, mosquito vector ecology and transmission seasonality. It is suggested that unknown factors limit mixed *Plasmodium* species infections, and that mixed-species infections protect against severe *Plasmodium falciparum* malaria. Careful examination of methods used to detect these parasites and interpretation of individual- and population-based data are necessary to understand the influence of mixed *Plasmodium* species infections on malarial disease. This should ensure that deployment of future antimalarial vaccines and drugs will be conducted in a safe and timely manner.

Although novel exceptions have been reported [1], it is commonly agreed that *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale* are the four species that cause human malaria. Factors underlying mixed infections involving these parasite species have been discussed since 1930, when Knowles and White acknowledged difficulties that microscopists might encounter in accurately documenting their findings from examinations of blood smears [2]. Humans often harbor multiple *Plasmodium* species [3–6], and varying patterns in species-specific parasitemia and mixed-species prevalence characterize malaria infections in different endemic regions ([7–10; reviewed in Refs [11,12]). Data from malaria fever therapy patients involving different *Plasmodium* species [13–15], and naturally infected study volunteers, have illustrated a range of observations from orderly to turbulent species-specific patterns (Figure 1) of parasitemia in infected individuals [13–17]. Antimalarial treatment studies have also contributed insight regarding mixed *Plasmodium* species infections by revealing undocumented infection of a second species following successful treatment of a first species (reviewed in Ref. [18]).

These varied patterns of parasitemia observed in individuals infected by multiple *Plasmodium* species are surely influenced by a complex array of host factors [19] acting to constrain the infection before the parasites completely over-run the available erythrocyte population. Evidence that the four *Plasmodium* parasites of humans antagonize one another seems less clear.

Conflicting results suggesting the presence or absence of mixed-species interactions from one study to the next are seldom resolved. If direct interaction between *Plasmodium* species occurs within an infected individual to any significant level, these interactions would then be expected to influence the distribution of species within the endemic population. A common theme discussed in the context of *Plasmodium* species interactions has focused on suppression of mixed infections. However, suppression of mixed-species infections is only one of the patterns found to characterize infection by multiple *Plasmodium* species in endemic human populations, as summarized by Richie [11]:

'As yet, no consistent evolutionary relationship between the species of human malaria parasites has emerged. I am led to conclude that there are geographical differences in the way in which human malaria species interact, and that these interactions may even change from year to year in a given locale. In summary, the strongest statement that can be made from the available data are that suppression or exclusion may occur between malaria parasites, but that these effects may be masked, particularly in regard to the prevalence of mixed infections, by factors such as heterogeneity in host susceptibility.'

It is possible that interactions among *Plasmodium* species infecting humans can influence efforts to develop future successful malaria control strategies in the ecologically diverse malaria-endemic settings. Thus, it is important to review the status of tools used to characterize malaria infections, as well as the interpretation of data generated by these tools and some of the practical issues that will continue to confront malaria control efforts. Identification of existing gaps in diagnostic technology, and consequently our knowledge of dynamic features of malaria infection (Box 1), will permit evaluation of how current limitations might influence our understanding of mixed *Plasmodium* species infections in humans.

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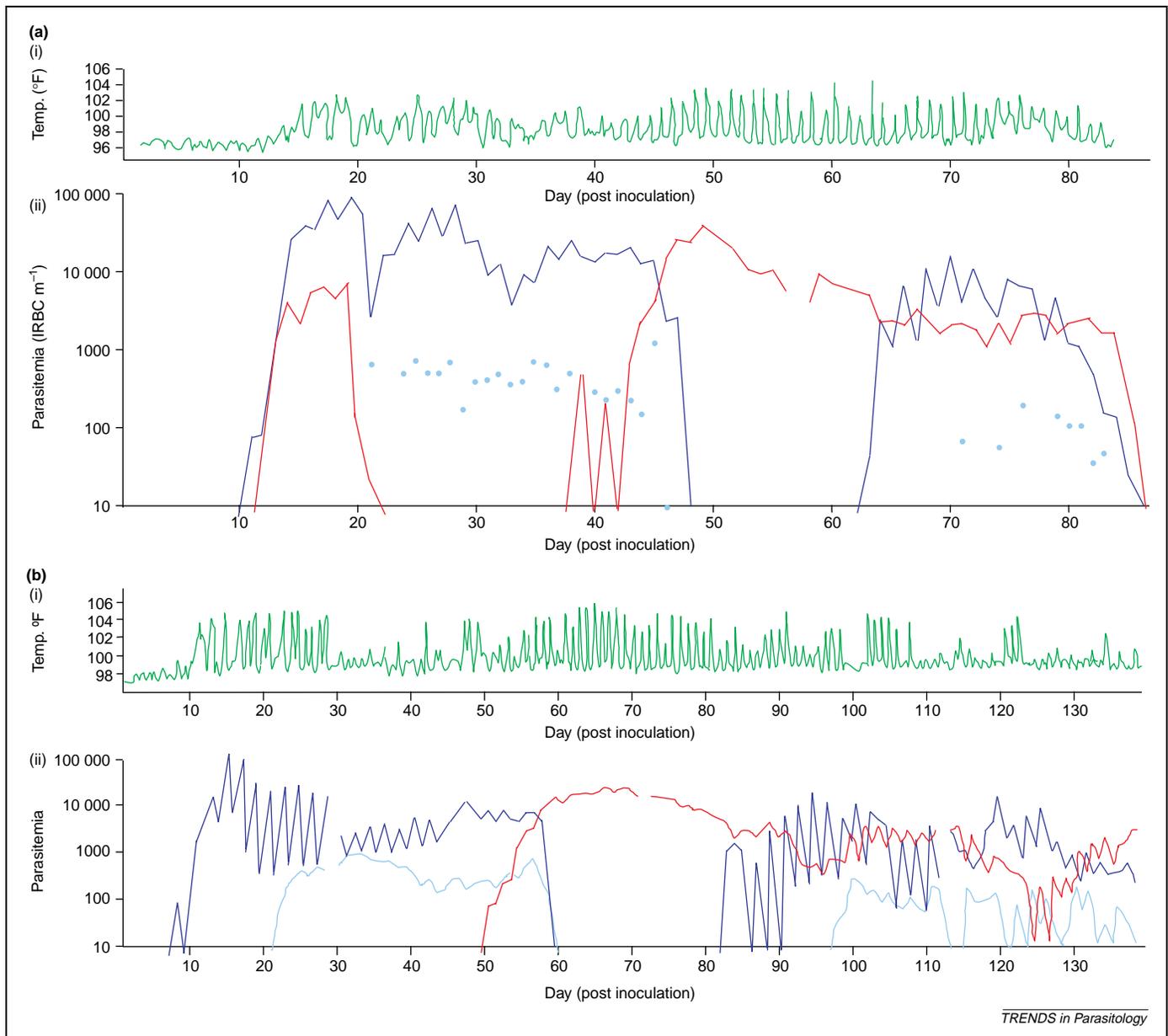
Figure 1. Adaptation of clinical summaries originally presented by Boyd and Kitchen from two patients receiving *Plasmodium falciparum* and *Plasmodium vivax* simultaneously. Each record shows the natural history of the patient's temperature (green line) and blood-smear parasitemia (no. of parasitized erythrocytes per μl) monitored daily. **(a)** The data obtained from Ref. [13] exhibits a fairly regular pattern of *P. falciparum* (blue line) and *P. vivax* (red line) parasitemia where numerous consecutive blood smears detected only one species; *P. falciparum* gametocytes are represented by light blue dots. **(b)** Data obtained from Ref. [14] exhibits a similar (above) mixed infection pattern until Day 83. Following this time-point, both species were equally prominent in the blood smears and could represent chronic infections observed in individuals from malarious regions. Reproduced, with permission, from Refs [13,14].

Box 1. Knowledge gaps underlying the mixed *Plasmodium* species infection debate

Gap 1: Diagnosis of malaria infection (particularly low-level infections) remains a growing challenge [20,21]. What diagnostic methods are best for performing studies of this nature? How accurate are laboratory techniques in their species-specific quantitative assessments?

Gap 2: Dynamic fluctuation of *Plasmodium* species is observed in infected individuals and malaria-endemic communities. What factors contribute to these fluctuations? Is there something that one *Plasmodium* species triggers to influence infection by a second, third or fourth species? How does the dynamic flux in mixed-species parasitemia within an individual translate into patterns of infection within the endemic population? If there is a dominant species, would it replace the less-dominant species?

Gap 3: Functionally similar merozoite surface proteins exist and participate in erythrocyte invasion [61]. Do antigenic similarities exist among the human malaria parasite surface proteins? What are the implications of antigenic similarity on developing vaccines that target more than one *Plasmodium* species? What are the implications of antigenic similarity on mixed *Plasmodium* species interactions?

Gap 4: Mixed *Plasmodium* species interactions have been suggested to influence clinical disease [65–68]. If species interactions reduce the severity of malaria illness, will vaccine or drug development programs targeting one species disrupt an important balance in human infection and increase the risk of severe disease in endemic populations?

Plasmodium species diagnosis

Blood-smear diagnosis is the most widely utilized approach for generating malaria infection data for epidemiological studies focused on mixed-species infections. In addition, PCR-based methodologies have introduced new strategies for malaria diagnosis worthy of consideration. Both techniques encounter qualitative and quantitative limitations.

The blood-smear produces quantitative information and the range of parasitemia detected generally corresponds with clinical malaria. However, blood-smear microscopy reaches its limit of detection when parasitemia falls below 40 infected red blood cells (IRBC) per microliter of blood (10^8 total body parasites; Box 2), and the reproducibility of parasite counts and species identification is frequently inconsistent [20,21]. Factors influencing the precision of blood-smear diagnosis include the quality of the blood slide preparation, the number of microscope fields analyzed (blood volume), and the microscopists' expertise. Furthermore, in regions of hypo- to meso-endemicity (Box 3), low parasitemia (<5 IRBC μl^{-1}) can make species identification difficult. Each of these factors influence studies on mixed *Plasmodium* species infection and will affect diagnostic results from different endemic regions in different ways. These practical issues make it difficult to compare results of studies on mixed *Plasmodium* species infections within and between endemic regions.

In well-equipped laboratories, conventional PCR diagnosis of malaria is less constrained by operator expertise. The methods can be performed on hundreds of samples at a time in automation-ready formats (96-well plates), on samples archived for years under varying storage conditions, and encounters limits of detection only when parasitemia falls below 0.5 IRBC μl^{-1} (10^6 total body parasites). As mass production of sample processing introduces elements that improve uniformity of analysis, DNA extractions, PCR and detection reactions performed in 96-well plates using reagents prepared in volumes accommodating thousands of analyses favor improved precision and reproducibility of *Plasmodium* species diagnosis. Although PCR-based data have significantly changed perspectives on malaria epidemiology [3,6,22,23], diagnosis by these more-sensitive strategies does have limitations, for example: (i) PCR-based assays require expensive equipment and large quantities of disposable supplies; (ii) contamination can contribute to false positive results; (iii) until recently, PCR diagnostic assays have not provided species-specific enumeration of parasites; and (iv) DNA-based diagnostic strategies do not differentiate among the various developmental stages within infected erythrocytes and are not likely to detect blood-stage infection at its first onset (parasitemia, 5×10^{-3} IRBC μl^{-1}; 10^4 total body parasites) because of the sample volume assayed ($\sim 1 \mu l$). Real-time PCR methodologies [24] (and other DNA-based strategies [25]) provide quantitative data on the amount of species-specific templates within a sample; however, optimizing these diagnostic strategies requires significant expertise.

Alternative assays based upon commercially developed antigen-capture test kits have been designed primarily to diagnose *P. falciparum*. Target antigens include histidine-rich protein 2 (HRP-2) [26] or *Plasmodium* lactate dehydrogenase (pLDH; live parasites) [27]. These assays are quick and easy to perform. However, limitations of the antigen-capture tests are encountered as they do not enable assessment of parasitemia and do not detect *P. vivax*, *P. malariae* or *P. ovale* specifically. Additionally, these assays are frequently less sensitive than microscopy, and can produce false positivity through detection of persistent antigenemia following parasite clearance (HRP-2) and through crossreactivity between HRP-2 and rheumatoid factor [28].

Finally, *P. falciparum* presents unique challenges for enumerating blood-stage parasitemia in the infected human host because late-stage trophozoites and schizonts sequester in post-capillary venules. Some studies estimate that $>75\%$ of *P. falciparum*-infected erythrocytes are sequestered in the peripheral vasculature [29,30]. Uncertainty related to the true level of *P. falciparum* sequestration could significantly influence quantitative estimates of species-specific parasitemia and present important challenges to modeling and interpretation of mixed *Plasmodium* species infections.

Infection dynamics

Human infection studies such as those performed during the era of the neurosyphilis trials [31–33] are not repeatable [34]; however, these early patient studies contributed significantly to our ability to work with malaria parasites in research laboratories over the past 75 years, as well as to our understanding of the basic characteristics of human malaria infection [35]. To interpret mixed *Plasmodium* species infections, basic biological characteristics of infection by each species must be considered (Figure 2). The minimum duration of liver-stage infection is 6 days (*P. falciparum*) [36], 8–9 days (*P. vivax* and *P. ovale*) [36,37] and 15 days (*P. malariae*) [38]. It is known that *P. vivax* produces a 'dormant' liver-stage infection through developmentally

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Box 2. Approximate hematological and parasitemic quantities

Although normal hematological values vary with age, sex, ethnicity and health status, a discussion regarding mixed-species interactions requires quantitative reference points to enable comparisons to detect significant deviations away from a null hypothesis of no interaction. The following 'working values' are provided for this purpose.

Normal hematological values
5×10^6 erythrocytes per microliter (μl) of blood
8×10^3 leukocytes per microliter (μl) of blood

2.5×10^{13} erythrocytes per total adult body

Parasitemia by blood smear
For a conventional blood smear, infected red blood cells (IRBC) are counted in microscopy fields containing 200 leukocytes (1/40th of a μl). To approximate parasitemia per μl, multiply IRBC by 40.

Limit of blood smear sensitivity
1 IRBC per 200 leukocytes $= 40$ IRBC per $\mu l = 2 \times 10^8$ total body parasites

Box 3. Malaria endemicity

Assessing levels of malaria endemicity is becoming the work of theoretical modeling based upon seasonal or annual variation in climate, vegetation, malaria prevalence, environmental factors that influence mosquito breeding and feeding behaviors, and human population data [69]. Field-based malariometric studies have employed the following spleen enlargement and/or parasite prevalence scale [72].

Holoendemic: Spleen or parasite prevalence >75% and prevalence of adult spleen enlargement is low. Parasite rates in the first year of life are high.

Hyperendemic: Spleen or parasite prevalence 50–75% and prevalence of adult spleen enlargement is also high.

Mesoendemic: Spleen or parasite prevalence 10–50%.

Hypoendemic: Spleen or parasite prevalence 0–10%.

arrested hypnozoites [39]. Relapses of *P. vivax* and *P. ovale* blood-stage infections, frequently observed to occur months after the primary blood-stage infections have resolved, are thought to result from activation of this specialized life-cycle stage [36,39]. Curiously, although *P. malariae* is not known to produce hypnozoites, blood-stage infection by this species has been shown to re-emerge following years of blood-smear negativity [36,40]. The estimated number of merozoites produced by an infected hepatocyte varies from 30 000 (*P. falciparum*) to 10 000 (*P. vivax*) and 15 000 (*P. malariae* and *P. ovale*) [36].
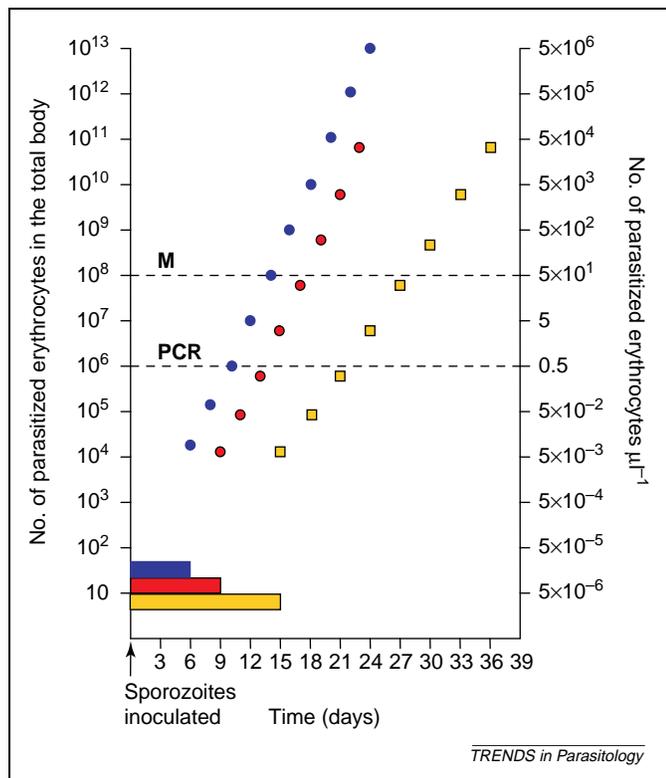
Figure 2. The characteristics of pre-erythrocytic and erythrocytic phases of parasitemia for different malaria species of humans. The blood smear kinetics shown here assume that a single sporozoite from different malaria species were used to infect humans, as indicated: *Plasmodium falciparum* (blue bar and circle), *Plasmodium vivax* (red bar and circle); *Plasmodium ovale* resembles *P. vivax*; and *Plasmodium malariae* (yellow bar and square). The minimum duration of liver-stage infection is represented by blue (*P. falciparum*), red (*P. vivax* and *P. ovale*), and yellow (*P. malariae*) bars. Key: E, elimination; M, parasitemia when malaria infection will be observed by conventional blood-smear analysis (2×10^8 total number of parasites in the body); PCR, parasitemia when malaria infection will be observed by PCR-based diagnosis (1 infected erythrocyte per 5×10^6 erythrocytes μl^{-1}).

Plasmodium species have been observed to exhibit target cell population preferences. Whereas *P. falciparum* shows a preference for younger erythrocytes, it is capable of infecting erythrocytes of all ages [41]. By contrast, *P. vivax* and *P. ovale* are observed to prefer infection of reticulocytes [36,42], whereas *P. malariae* is suggested to prefer infection of mature erythrocytes [41]. During asexual blood-stage replication, the human *Plasmodium* species parasites produce tens of merozoites per infected cell (*P. falciparum* mean of 16, range 8–32; *P. vivax* mean of 16, range 12–24; *P. malariae* mean of 8, range 8–12; *P. ovale* mean of 8, range 4–16 [36]). The pattern of fever and duration of the erythrocyte development cycle vary from 48 h (*P. falciparum*, *P. vivax* and *P. ovale*) to 72 h (*P. malariae*).

The parasite density provoking a fever (>37.3 °C) [43], known as the pyrogenic density (PD), varies considerably from species to species: *P. vivax* and *P. ovale* induce fever at parasitemias around 100 IRBC μl^{-1} in non-immune adults [32], and *P. malariae* induces fever at a parasitemia of around 500 IRBC μl^{-1} [43]. By contrast, *P. falciparum* induces fever at higher parasitemias of around 10^4 IRBC μl^{-1} [32,43]. The parasitemia of *P. falciparum* infection is estimated to reach levels ~100-fold higher than the other three human malaria parasite species and may exceed 10^5 IRBC μl^{-1} [32]. Whereas these values are observed to vary among endemic settings, across age ranges (PD decreases with age), and with level of malaria exposure [9,44–46], a consistent finding that the PD for *P. falciparum* is at least tenfold higher than the PD for *P. vivax* has been reported [9,43]. As fever has been strongly associated with parasite killing in non-immune individuals [32], this host response mechanism is considered to play an important role in regulating the rise and fall of parasitemia in infected individuals [47]. Interestingly, it has been suggested that the association between fever and changes in parasitemia may not be as strong in immune individuals [48].

Examination of the salient quantitative differences distinguishing the human *Plasmodium* parasite species suggests that the 'playing field' is not level when these parasites compete for available erythroid target cells. On the basis of the species-specific characteristics reviewed above, the intra-individual infection landscape might appear to favor *P. falciparum* over the other *Plasmodium* species, and could translate into fluctuations between predominant and minority species within an endemic population. Although studies by Desowitz [49] and Cattani [50] provide evidence that *P. falciparum* overtakes the other malaria parasite species at a population level in Papua New Guinea (PNG), the evolution of chloroquine resistance in *P. falciparum* could have been the most important factor underlying this change. Despite this shift, and the other factors that might have been involved, the curious fact remains that the other three human malaria parasites are all commonly observed in infected individuals in PNG [6,8,23]. In contrast to *Plasmodium* species shifts in PNG, the prevalence of *P. vivax* malaria in South America has been increasing in recent years despite the co-prevalence of *P. falciparum* [51]. Therefore, the relationships between intra-individual and population-based changes among the human *Plasmodium* parasite

species present further challenges that must be understood as vaccines and new strategies to control malaria are developed.

A recent study [16] provides a new look at intra-individual *Plasmodium* species relationships through a longitudinal analysis of mixed-species infections in 34 children (ages 4–14 years) living near Madang, PNG. Beyond self-evident differences with malaria fever therapy studies, ≈50% of the PNG children were blood-smear-positive for at least one *Plasmodium* species on the first day of the study, and so it is likely that all participants had experienced infection by one of the four human malaria species before the study. In addition, blood smears and clinical symptoms of the PNG children were evaluated on three-day intervals, not daily as shown in Figure 1. The data from this PNG study provide examples showing that, when blood-smear parasitemia met with density-dependent constraints at or around 1000 parasites μl^{-1} (near the fever threshold in the same study population [44]), parasitemia often, although not always, went below blood-smear microscopy detection limits or resulted in a switch in the species formula (Pf:Pv:Pm) [16]. Bruce *et al.* interpret these findings to suggest that a relatively stable infection formula persists until a majority parasite population breaks through the threshold. At this point in the infection, mechanisms underlying a density-dependent regulatory force act against all infecting *Plasmodium* species and send the total parasitemia to some level below the threshold. Afterwards, the regulatory force is switched 'off' and the parasite populations resume growth and replication. Although the biological mechanism(s) favored in this species-transcending density-dependent (STDD) regulation of malaria infection include components of the innate immune system augmented by elements of acquired immunity against former majority populations, it is also important to consider the influence of several host genetic polymorphisms when interpreting any individual's infection status or parasitemia.

Integrating what might be the expected influence of a STDD regulatory force on mixed-species infections and the distribution of parasites within endemic populations requires further consideration as the impact and durability of this force might affect what is observed in prevalence surveys. For example, if activation of the STDD force had a strong impact (Figure 3a) on the parasite population, it could drive the frequency of blood-smear and PCR positivity below levels of detection, or eliminate infection. It has been, and continues to be, difficult for diagnostic methodologies to determine to what levels parasitemia falls (Figure 1). Depending on the species-specific parasitemia following deactivation of the force, many more non-infected and single-species infections compared with mixed-species infections may be observed within the endemic population, consistent with the 'deficit' of mixed species infections reported by some [16,52]. Alternatively, if the force is modest (Figure 3b), parasitemia might not fall below microscopy and PCR detection limits, the combination of parasites infecting individuals might not change, and a higher number of mixed species infections would be found in the population. Although the data presented by Bruce *et al.* [16,48,53]

illustrate that the STDD force is consistently durable in keeping parasitemia below 1000 IRBC μl^{-1}, study participants were seldom free of infection and PCR data suggested that infection complexity was higher by PCR than was reported by blood-smear diagnosis [16,54].

This latter interpretation of the STDD force would be consistent with our data from three individual malaria prevalence surveys [6,23] conducted in Madang (Liksul) and East Sepik (Dreikikir and Wosera) Provinces in PNG. Evaluating 1242–2162 individuals, we compared numbers of non-infected, single-species infected and mixed-species infected. Although we observed a deficit of mixed-species infections (single-species outnumbered mixed-species infections) by blood-smear, this deficit was reversed or substantially diminished when a subset of the population was evaluated by PCR [6,23]. Furthermore, for both blood-smear- and PCR-based diagnostic results, chi-square analyses indicated that mixed-species infections in all three study populations were detected at, and not below (as might be implied by a deficit), their expected frequencies. This distribution of *Plasmodium* species within the endemic populations studied did not differ from an expected random distribution pattern and suggests no cross-species interactions. These studies, conducted in similar malaria-endemic regions of PNG, have focused on different aspects of mixed-species infections (intra-individual [16] and community-wide [6,23]) and have reached different conclusions regarding cross-species interactions. Results appear to suggest that interactions among *Plasmodium* species within individuals do not influence the community-wide prevalence and distribution of *Plasmodium* species. From this, important practical questions arise in relation to vaccine development and evaluation of clinical malaria in regions where multiple *Plasmodium* species infect humans (Box 1, Gap 3 and Gap 4).

Immunological crossreactivity

Although early studies investigating mixed *Plasmodium* species interactions suggested that it might be possible for antigenic similarities between species to allow an individual vaccine molecule derived from one species to offer protection from the other human malaria parasites [55], data to support this hypothesis have not been produced [56]. In fact, little if any cross-species immunity was observed during the malaria fever therapy trials, and individuals exposed to one *Plasmodium* species did not exhibit protection from high parasitemia or clinical malaria resulting from infection by a different *Plasmodium* species [33,36,57]. Primary *P. malariae* infection was associated with lower parasitemia and clinical disease in secondary *P. falciparum* malaria [33]. Reasons contributing to the illusive nature of cross-species immunity have become apparent, as low sequence similarity does not promote cross-*Plasmodium* species immune recognition despite similar antigen function, expression and localization. More specifically, studies characterizing the circumsporozoite proteins (CSPs) of human malaria parasites reported many amino acid sequence differences among *P. falciparum* (primary repeating amino acid motif NANP) [58], *P. vivax* (GDRADGQPA [VK210], ANGAGNQPG

Figure 3. Further consideration that a species-transcending density-dependent force regulates mixed *Plasmodium* species infections. A summary of the proportion of each *Plasmodium* species is given in **(ai)** and **(bi)**, observed by blood smear corresponding to simulated data for each of 12 three-day sample collection time points [indicated in **(aii,bii)**] Hypothetical data predicted to occur by asexual replication characteristics of the human *Plasmodium* species parasites and principles proposed in the species-transcending density-dependent (STDD) model. Orange shading represents parasite density above a threshold of ∼1000 parasitized erythrocytes per μl (T). Gray shading identifies the limits of detection by microscopy (M). The black broken line represents the limits of detection by PCR. Acquired immunity is indicated by arrows. Asexual replication of the different *Plasmodium* species is shown as colored solid lines and parasite killing as colored broken lines. Key: purple, *Plasmodium falciparum* strain A; gray bars, no parasites; light blue, *P. falciparum* strain B; red, *Plasmodium vivax*; yellow; *Plasmodium malariae*.

[VK247]) [59], and *P. malariae* (NAAG) [60]. More recently, comparative studies have observed little homology among erythrocyte-binding proteins [61]. Specifically, del Portillo *et al.* reported that, although 17 out of 22 cysteine residues were similarly positioned between the *P. vivax* and *P. falciparum* merozoite surface antigens (MSA1/MSP1), there was only 35.6% amino acid identity between these species [62]. With continued progress on sequencing the *Plasmodium* species genomes [63,64], it might be possible for previously unidentified molecular homology between species to be identified and alter this working perspective.

Impact of mixed-species infection on malarial disease
Several different studies have now reported that *P. vivax* infections help to reduce the severity of *P. falciparum* malaria [65–68]. The study conducted in Vanuatu [65] suggested that α⁺ thalassemia might predispose young children to the more 'benign' *P. vivax* infection, which proves beneficial later when children become most susceptible to severe *P. falciparum* malaria. In studies from Thailand, Luxemburger *et al.* [66] reported that severe *P. falciparum* malaria was reduced from 5.7% (293 out of 5148) in patients infected with *P. falciparum* alone to 1.6% (10 out of 628) in patients with a *P. falciparum*–

P. vivax co-infection. These authors suggest that fever induced at lower parasitemia by *P. vivax* (PD of ∼200 parasites per μl for *P. vivax*; 1500 parasites per μl for *P. falciparum*) might limit parasitemia and the pathogenic potential of *P. falciparum*. Smith *et al.* [67] have also suggested that *P. vivax* appeared to protect against *P. falciparum* disease in their studies in the Wosera region of PNG.

In addition, Price *et al.* [68] suggest that those with *P. falciparum*–*P. vivax* co-infection in Thailand showed less-severe anemia compared with individuals infected with *P. falciparum* alone. Although we have made similar observations in the Wosera (D. Tisch *et al.*, unpublished), *P. vivax* contributed equally to anemia when compared with *P. falciparum* in single-species infections. As anemia is responsible for significant morbidity, and malaria is the second leading cause of hospitalization and death in PNG, it is difficult to conclude that constraints should be made against control of *P. vivax* malaria. As results of clinical malaria studies have largely been based upon blood-smear diagnosis of malaria infections, a more-sensitive diagnostic assay could reduce or nullify the apparent clinically protective effects associated with mixed *Plasmodium* species infections.

Conclusion

We need to know more about mixed *Plasmodium* species infections because we know so little about the mechanisms regulating innate and acquired immunity against malaria in children under five years old, who bear the greatest risk of disease. As malaria affects humans at individual and population levels differently across environmentally varying regions [69], it is important to encourage continued survey of malaria through both prevalence and longitudinal studies, and to improve diagnostic techniques so that species-specific parasitemia can be determined more efficiently and with greater precision. It is also important that assays capable of evaluating a wide range of immunological effector mechanisms from small amounts of blood should be developed. Along with improvement of laboratory methods for analyzing blood samples, any malaria survey study design should emphasize the importance of close interval sampling. Studies of this nature are likely to provoke discussions regarding the ethical conduct of research on vulnerable populations, particularly in individuals classified as 'non-immune'. However, without these studies, our understanding of the biological mechanisms that regulate mixed *Plasmodium* species infections in these regions will remain limited. These limitations have the potential to influence decisions on testing vaccines and new antimalarial drugs. Because clinical studies suggest that *P. vivax* infection in co-endemic regions might decrease levels of *P. falciparum* parasitemia and severe pathogenesis, assumptions may follow that trials and deployment of *P. vivax*-specific vaccines or drugs [18,70,71] could lead to severe consequences of increased morbidity and mortality from *P. falciparum* malaria in treated regions. As many studies show that *P. vivax* represents a significant public health burden, forestalling tests on *P. vivax*-specific vaccines and drug strategies where mixed-species infections occur might prolong the impact of this parasite unnecessarily.

References

1 Singh, B. *et al.* (2004) A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. *Lancet* 363, 1017–1024

2 Knowles, R. *et al.* (1930) Studies in the parasitology of malaria. *Indian Medical Research Memoirs* 18, 436

3 Snounou, G. *et al.* (1993) Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Mol. Biochem. Parasitol.* 58, 283–292

4 Zhou, M. *et al.* (1998) High prevalence of *Plasmodium malariae* and *Plasmodium ovale* in malaria patients along the Thai–Myanmar border, as revealed by acridine orange staining and PCR-based diagnoses. *Trop. Med. Int. Health* 3, 304–312

5 Purnomo, A. *et al.* (1999) Rare quadruple malaria infection in Irian Jaya Indonesia. *J. Parasitol.* 85, 574–579

6 Mehlotra, R.K. *et al.* (2000) Random distribution of mixed species malaria infections in Papua New Guinea. *Am. J. Trop. Med. Hyg.* 62, 225–231

7 Molineaux, L. *et al.* (1980) A longitudinal study of human malaria in the West African Savanna in the absence of control measures: relationships between different *Plasmodium* species, in particular *P. falciparum* and *P. malariae*. *Am. J. Trop. Med. Hyg.* 29, 725–737

8 Genton, B. *et al.* (1995) The epidemiology of malaria in the Wosera area, East Sepik Province, Papua New Guinea, in preparation for vaccine trials. I. Malariometric indices and immunity. *Ann. Trop. Med. Parasitol.* 89, 359–376

9 Luxemburger, C. *et al.* (1996) The epidemiology of malaria in a Karen population on the western border of Thailand. *Trans. R. Soc. Trop. Med. Hyg.* 90, 105–111

10 Postigo, M. *et al.* (1998) Malaria diagnosis by the polymerase chain reaction: a field study in south-eastern Venezuela. *Trans. R. Soc. Trop. Med. Hyg.* 92, 509–511

11 Richie, T.L. (1988) Interactions between malaria parasites infecting the same vertebrate host. *Parasitology* 96, 607–639

12 McKenzie, F.E. and Bossert, W.H. (1997) Mixed-species *Plasmodium* infections of humans. *J. Parasitol.* 83, 593–600

13 Boyd, M.F. and Kitchen, S.F. (1938) Vernal vivax activity in persons simultaneously inoculated with *Plasmodium vivax* and *Plasmodium falciparum*. *Am. J. Trop. Med.* 18, 505–514

14 Boyd, M.F. and Kitchen, S.F. (1937) Simultaneous inoculation with *Plasmodium vivax* and *Plasmodium falciparum*. *Am. J. Trop. Med.* 17, 855–861

15 Jeffery, G.M. (1966) Epidemiological significance of repeated infections with homologous and heterologous strains and species of *Plasmodium*. *Bull. World Health Organ.* 35, 873–882

16 Bruce, M.C. *et al.* (2000) Cross-species interactions between malaria parasites in humans. *Science* 287, 845–848

17 Molineaux, L. *et al.* (2002) Malaria therapy reinoculation data suggest individual variation of an innate immune response and independent acquisition of antiparasitic and antitoxic immunities. *Trans. R. Soc. Trop. Med. Hyg.* 96, 205–209

18 Mayxay, M. *et al.* (2004) Mixed-species malaria infections in humans. *Trends Parasitol.* 20, 233–240

19 Good, M.F. *et al.* (1998) Pathways and strategies for developing a malaria blood-stage vaccine. *Annu. Rev. Immunol.* 16, 57–87

20 Anonymous (2000) *WHO/MAL/2000.1091New Perspectives in Malaria Diagnosis*, World Health Organization

21 Ohrt, C. *et al.* (2002) Impact of microscopy error on estimates of protective efficacy in malaria-prevention trials. *J. Infect. Dis.* 186, 540–546

22 Oliveira, D.A. *et al.* (1995) Polymerase chain reaction and a liquid-phase, nonisotopic hybridization for species-specific and sensitive detection of malaria infection. *Am. J. Trop. Med. Hyg.* 52, 139–144

23 Mehlotra, R.K. *et al.* (2002) Malaria infections are randomly distributed in diverse holoendemic areas of Papua New Guinea. *Am. J. Trop. Med. Hyg.* 67, 555–562

24 Farcas, G.A. *et al.* (2004) Evaluation of the RealArt Malaria LC real-time PCR assay for malaria diagnosis. *J. Clin. Microbiol.* 42, 636–638

25 Schoone, G.J. *et al.* (2000) Detection and quantification of *Plasmodium falciparum* in blood samples using quantitative nucleic acid sequence-based amplification. *J. Clin. Microbiol.* 38, 4072–4075

26 Shiff, C.J. *et al.* (1993) The rapid manual ParaSight-F test. A new diagnostic tool for *Plasmodium falciparum* infection. *Trans. R. Soc. Trop. Med. Hyg.* 87, 646–648

27 Palmer, C.J. *et al.* (1998) Evaluation of the OptiMAL test for rapid diagnosis of *Plasmodium vivax* and *Plasmodium falciparum* malaria. *J. Clin. Microbiol.* 36, 203–206

28 Moody, A. (2002) Rapid diagnostic tests for malaria parasites. *Clin. Microbiol. Rev.* 15, 66–78

29 Silamut, K. and White, N.J. (1993) Relation of the stage of parasite development in the peripheral blood to prognosis in severe falciparum malaria. *Trans. R. Soc. Trop. Med. Hyg.* 87, 436–443

30 Gravenor, M.B. *et al.* (2002) A model for estimating total parasite load in falciparum malaria patients. *J. Theor. Biol.* 217, 137–148

31 O'Leary, P.A. (1927) Treatment of neurosyphilis by malaria: report on the three years' observation of the first one hundred patients treated. *J. Am. Med. Assoc.* 89, 95–100

32 Kitchen, S.F. (1949) Symptomatology: general considerations. In *Malariology; A Comprehensive Survey of all Aspects of This Group of Diseases from a Global Standpoint* (Boyd, M.F. ed), pp. 966–994, W.B. Saunders

33 Collins, W.E. and Jeffery, G.M. (1999) A retrospective examination of sporozoite- and trophozoite-induced infections with *Plasmodium falciparum* in patients previously infected with heterologous species of *Plasmodium*: effect on development of parasitologic and clinical immunity. *Am. J. Trop. Med. Hyg.* 61 (Suppl 1), 36–43

34 McCarthy, M. (2003) US researcher broke federal rules in aiding Chinese HIV study. *Lancet* 361, 1528

35 Chernin, E. (1984) The malariatherapy of neurosyphilis. *J. Parasitol.* 70, 611–617

36 Garnham, P.C.C. (1966) *Malaria Parasites and Other Haemosporidia*, Blackwell Scientific Publications

37 Fairley, N.H. (1947) Sidelights on malaria in man obtained by subinoculation experiments. *Trans. R. Soc. Trop. Med. Hyg.* 40, 621–676

38 Coatney, G.R. *et al.* (1971) *The Primate Malarias*, National Institute of Allergy and Infectious Diseases

39 Krotoski, W.A. *et al.* (1982) Demonstration of hypnozoites in sporozoite-transmitted *Plasmodium vivax* infection. *Am. J. Trop. Med. Hyg.* 31, 1291–1293

40 Vinetz, J.M. *et al.* (1998) *Plasmodium malariae* infection in an asymptomatic 74-year-old Greek woman with splenomegaly. *N. Engl. J. Med.* 338, 367–371

41 Kitchen, S.F. (1939) The infection of mature and immature erythrocytes by *Plasmodium falciparum* and *Plasmodium malariae*. *Am. J. Trop. Med.* 19, 47–62

42 Kitchen, S.F. (1938) The infection of reticulocytes by *Plasmodium vivax*. *Am. J. Trop. Med.* 18, 347–353

43 White, N.J. (2003) Malaria. In *Manson's Tropical Diseases* (Zumla, A. and Cook, G.C. eds), pp. 1205–1295, W.B. Saunder Co. Ltd

44 Cox, M.J. *et al.* (1994) Dynamics of malaria parasitaemia associated with febrile illness in children from a rural area of Madang, Papua New Guinea. *Trans. R. Soc. Trop. Med. Hyg.* 88, 191–197

45 Rogier, C. *et al.* (1996) Evidence for an age-dependent pyrogenic threshold of *Plasmodium falciparum* parasitemia in highly endemic populations. *Am. J. Trop. Med. Hyg.* 54, 613–619

46 Gatton, M.L. and Cheng, Q. (2002) Evaluation of the pyrogenic threshold for *Plasmodium falciparum* malaria in naive individuals. *Am. J. Trop. Med. Hyg.* 66, 467–473

47 Gravenor, M.B. and Kwiatkowski, D. (1998) An analysis of the temperature effects of fever on the intra-host population dynamics of *Plasmodium falciparum*. *Parasitology* 117, 97–105

48 Bruce, M.C. and Day, K.P. (2002) Cross-species regulation of malaria parasitaemia in the human host. *Curr. Opin. Microbiol.* 5, 431–437

49 Desowitz, R.S. and Spark, R.A. (1987) Malaria in the Maprik area of the Sepik region, Papua New Guinea: 1957–1984. *Trans. R. Soc. Trop. Med. Hyg.* 81, 175–176

50 Cattani, J.A. *et al.* (1986) The epidemiology of malaria in a population surrounding Madang, Papua New Guinea. *Am. J. Trop. Med. Hyg.* 35, 3–15

51 Anonymous. (2001) Situation of Malaria Programs in the Americas. *Epidemiol. Bull.* 22, 10–14

52 Maitland, K. *et al.* (1996) The interaction between *Plasmodium falciparum* and *P. vivax* in children on Espiritu Santo island, Vanuatu. *Trans. R. Soc. Trop. Med. Hyg.* 90, 614–620

53 Bruce, M.C. and Day, K.P. (2003) Cross-species regulation of *Plasmodium* parasitemia in semi-immune children from Papua New Guinea. *Trends Parasitol.* 19, 271–277

54 Bruce, M.C. *et al.* (2000) Genetic diversity and dynamics of *Plasmodium falciparum* and *P. vivax* populations in multiply infected children with asymptomatic malaria infections in Papua New Guinea. *Parasitology* 121, 257–272

55 Cohen, J.E. (1973) Heterologous immunity in human malaria. *Q. Rev. Biol.* 48, 467–489

56 Richie, T.L. and Saul, A. (2002) Progress and challenges for malaria vaccines. *Nature* 415, 694–701

57 Boyd, M.F. *et al.* (1939) Consecutive inoculations with *Plasmodium vivax* and *Plasmodium falciparum*. *Am. J. Trop. Med.* 19, 141–150

58 Dame, J.B. *et al.* (1984) Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite *Plasmodium falciparum*. *Science* 225, 593–599

59 Rosenberg, R. *et al.* (1989) Circumsporozoite protein heterogeneity in the human malaria parasite *Plasmodium vivax*. *Science* 245, 973–976

60 Lal, A.A. *et al.* (1988) Structure of the circumsporozoite gene of *Plasmodium malariae*. *Mol. Biochem. Parasitol.* 30, 291–294

61 Adams, J.H. *et al.* (1992) A family of erythrocyte binding proteins of malaria parasites. *Proc. Natl. Acad. Sci. U. S. A.* 89, 7085–7089

62 del Portillo, H.A. *et al.* (1991) Primary structure of the merozoite surface antigen 1 of *Plasmodium vivax* reveals sequences conserved between different *Plasmodium* species. *Proc. Natl. Acad. Sci. U. S. A.* 88, 4030–4034

63 Gardner, M.J. *et al.* (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* 419, 498–511

64 Carlton, J. (2003) The *Plasmodium vivax* genome sequencing project. *Trends Parasitol.* 19, 227–231

65 Williams, T.N. *et al.* (1996) High incidence of malaria in alpha-thalassaemic children. *Nature* 383, 522–525

66 Luxemburger, C. *et al.* (1997) The epidemiology of severe malaria in an area of low transmission in Thailand. *Trans. R. Soc. Trop. Med. Hyg.* 91, 256–262

67 Smith, T. *et al.* (2001) Prospective risk of morbidity in relation to malaria infection in an area of high endemicity of multiple species of *Plasmodium*. *Am. J. Trop. Med. Hyg.* 64, 262–267

68 Price, R.N. *et al.* (2001) Factors contributing to anemia after uncomplicated falciparum malaria. *Am. J. Trop. Med. Hyg.* 65, 614–622

69 Snow, R.W. *et al.* (1999) A preliminary continental risk map for malaria mortality among African children. *Parasitol. Today* 15, 99–104

70 Arevalo-Herrera, M. and Herrera, S. (2001) *Plasmodium vivax* malaria vaccine development. *Mol. Immunol.* 38, 443–455

71 Tsuboi, T. *et al.* (2003) Transmission-blocking vaccine of vivax malaria. *Parasitol. Int.* 52, 1–11

72 Anonymous (1951) *Report of the Malaria Conference in Equatorial Africa, Kampala, 1950 (Report)*. World Health Organization

Roll Back Malaria: questioning the campaign

The Roll Back Malaria initiative (http://www.rbm.who.int/) was launched in 1998 with a pledge to slash the number of deaths from malaria in half by 2010. However, data from the World Health Reports 1999–2003 show that the annual number of malaria-induced deaths worldwide is now higher than it was when the campaign was first launched.

At this halfway stage, the question is now whether or not the initiative can be saved? The three main tools to fight malaria are already available – bednets, artemisinin-based combination therapy and insecticides – but their use needs to be made more widespread.

For more information, please go to: http://bmj.bmjjournals.com/cgi/content/full/328/7448/1086

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