



Vivax series:

The co-existence of *Plasmodium*: sidelights from falciparum and vivax malaria in Thailand

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It is rare to find human populations exposed to a single malaria parasite species – in most endemic areas, at least three *Plasmodium* species co-exist. Here, we briefly review mixed species infection in malaria, and discuss apparently disparate clinical and epidemiological observations of *Plasmodium falciparum* and *Plasmodium vivax*, now equally prevalent in Thailand, which suggest that an ‘entente cordiale’ between these two species might be beneficial both to parasites and humans. If this were the case, the influence of changes in the parasite formula in endemic areas on the burden of malaria would become an important element of study.

‘Even an adolescent malariologist knows that in human subjects with mixed infections one species will predominate particularly if the infections are introduced more or less simultaneously’. This statement, made by G. Robert Coatney at the Charles Franklin Craig Lecture in 1968 [1], sums up concisely a view long-held by malariologists about the subject of mixed malaria species infections. However, should one wish to consult the original data from which this opinion is derived for human infections, the vast malaria literature yields but a mere handful of original articles solely devoted to experimental observations [2–7], and a few more devoted to detailed longitudinal observations [8–14]. Retrospective analysis of hitherto unpublished relevant observations made in volunteers or neurosyphilis patients experimentally infected with malaria in the USA in the 1940–60s have become recently available [15,16]. The notion that interactions, including antagonism, occur between different *Plasmodium* species when these are brought together in the human host is nonetheless broadly consistent with epidemiological and clinical observations, and is sustained by more-robust evidence from *Plasmodium* infections in non-human primates, rodents or birds. These data were brought together in the first (and, to date, the only) extensive and creditable review of mixed-species malaria infections across all vertebrate hosts [17]. However, results from

the experimental infections conducted by T.L. Richie remain frustratingly confined to his PhD thesis (T.L. Richie, Interactions between malaria parasites in the vertebrate host, PhD thesis, University of Pennsylvania, 1985).

Out of sight...

Mixed species infections have been a relatively neglected field during the prolific early investigations on malaria. This was due, in part, to the debate on the existence of different *Plasmodium* species of humans. In the 1890s, Italian malariologists split these parasites into three species, whereas Alphonse Laveran championed the idea that they all belonged to a single species – the ‘unicity’ theory – with the parasites merely changing morphologically. The debate was finally laid to rest in the 1920s in large part thanks to observations made during malariotherapy (for example, see Refs [18,19]). The issue of mixed infections was first raised in the masterly and erudite review of malaria epidemiology composed in 1930 by Knowles and Senior White [20]. At this time, only three of the four malaria parasites that infect humans (*Plasmodium falciparum*, *Plasmodium vivax* and *Plasmodium malariae*) were considered, since universal acceptance of *Plasmodium ovale*, first described in 1922 [21] as a species separate from *P. vivax*, was only slowly and begrudgingly won [22]. Knowles and Senior White considered that the generally low figures for the prevalence of mixed species infections were grossly underestimated. The limitations of diagnosis by microscopic examination and the reliance on the results of cross-sectional surveys in the face of profound seasonal variation in species prevalence were put forward as the main reasons for the underestimates.

Further exploration of the consequences of mixed species infections attracted little attention, despite tantalizing evidence of antagonism between the species [4,6]. The fact that meaningful interpretation would require complex experimental protocols or lengthy, detailed field observations, both exacting substantial expense and manpower, no doubt contributed to a reluctance in pursuing this area of malariology. However, the main

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reason most probably lay in the demonstration that susceptibility to infection by one species of malaria was not obviated by previous or current infection by another, and that acquired immunity to one species, be it through a single infection or multiple infections with one or numerous strains of this species, does not significantly alter the susceptibility to acquire an infection by another species.

Although *sensu stricto* this view still holds today, challenges to this doctrine have been raised. For human infections, a review of induced malaria data obtained in the USA revealed that prior infection with one species appeared to influence the course of subsequent infections by a heterologous species [23]. The possibility that interactions among the parasite species might actually confer an advantage to the host was further raised by a statistical analysis of the mixed infection prevalence recorded in selected epidemiological surveys [24]. Experimental evidence of cross-immunity was obtained for some *Plasmodium* species in primates [25,26] and through extensive investigations of the influence of mixed infections on the course of malaria in rodents [27]. Varying degrees of heterologous immunity were observed, mostly leading to alterations in the course of infection but rarely abolishing the susceptibility to being infected by the challenge species. However, in rodents, experimental simultaneous inoculations of the two species were rarely conducted [28–30]. In most of the studies, the heterologous challenge was administered after recovery from the immunizing parasite species, a scheme followed in subsequent studies in rodents [31], which presumably reflects the aims of developing a malaria vaccine.

Interpretation of all of the above experiments must be tempered by the possibility that the parasites used might not be genetically homogeneous. Indeed, some isolates were later demonstrated to harbor different parasite species, which are often very difficult to distinguish morphologically and/or present in unequal numbers. These caveats were excluded in the most recent experimental study of mixed infections [32], as cloned parasite lines were used and specific molecular tools were employed to distinguish and quantify them [33]. Though simply descriptive of the course of infection and the overall mortality, these studies yielded reproducible alterations specific to the diverse paired-species combinations. The fact that no conceptual framework that would account for these patterns could be reasonably elaborated is testimony to our ignorance of the biological and immunological mechanisms that regulate the course of malaria infections.

An emerging interest

Over the past decade, interest in the question of mixed-species *Plasmodium* infections has revived. This has been sparked by the introduction of sensitive molecular tools of detection, and sustained by the realization by a growing number of researchers that *Plasmodium* species other than *P. falciparum* are worthy of study. The first conference entirely devoted to *P. vivax* ('Vivax Malaria Research: 2002 and Beyond', convened by the Multilateral Initiative on Malaria in February 2002 in Bangkok, Thailand), which is possibly the most widespread of the

human parasites, is eloquent testimony of this revival. It is hoped that any perception that *P. vivax* is relatively clinically unimportant would be thereby corrected.

Two factors have hampered epidemiological studies of mixed infections in humans: the inadequacy of microscopic examination for detection, and the unavoidable necessity of repeated sampling over long periods. Both are inherent to the nature of the malaria infections, namely month- or year-long low-level chronic parasitemias, only occasionally disrupted by recrudescences that often remain clinically silent. Harnessing the power of PCR amplification to *Plasmodium* substantially diminished the first obstacle, and the ability to apply this technique to relatively small amounts of blood somewhat alleviates the intractable second obstacle.

Species-specific sequences present in the genes of the small subunit rRNA (ssrRNA) genes were used to develop protocols for the accurate identification of the four species that infect humans with a sensitivity close to one parasite per aliquot analyzed [34–36]. The first cross-sectional studies conducted with these tools [34,35,37] confirmed the opinions expressed by Knowles and Senior White 60 years ago – that the prevalence of mixed infections is greatly underestimated by microscopy. Similar observations for other diverse endemic areas using equivalent methodologies, gratifyingly almost invariably based on the parasites' ssrRNA genes, have clearly established mixed species infections as a common, if not the natural, state of affairs in humans.

Mixed infections further gained visibility when the possibility that concurrent infections with two *Plasmodium* species might modulate the pathology of the infection was raised by independent observations in the Ivory Coast [38] and in Sri Lanka [39]. These echoes of clinically beneficial associations between parasite species were amplified when the higher incidence of *P. vivax* in young α -thalassemic children in Vanuatu was proposed to confer an improved clinical immunity to subsequent *P. falciparum* infections [40,41]. Recently, the concept that some form of cross-species regulation of parasitemia occurs was elaborated from a detailed longitudinal study of asymptomatic infections in Papua New Guinean children [42–44]. Finally, relevant clinical and epidemiological observations have been recently collated in a review of mixed *Plasmodium* species infections in humans [45].

Efforts to unravel the mysteries of mixed species infection now profit from the elaboration of dedicated mathematical models pioneered by McKenzie [46–50]. Interpretations that are based, ultimately, on partial epidemiological data thereby benefit from a rigorous check and possibly novel perspectives.

A natural experiment

Clinical and epidemiological observations in Thailand, more than in any other endemic setting, have brought malaria mixed infections to the attention of malariologists. Thailand is particularly well suited to provide insights on interactions between *Plasmodium* species for several reasons. Thailand boasts excellent country-wide epidemiological surveillance and hosts several continuously monitored sites. Data are available for numerous patients

recruited for drug efficacy studies who have been observed for extended periods in a setting where malaria transmission does not occur (a comprehensive list of relevant studies can be found in Ref. [45]). Last, but by no means least, *P. falciparum* and *P. vivax* are the dominant species in the country and, over the past 10 years, the number of recorded cases have been nearly equally divided between these two species [51]. It is interesting to note the dominance of *P. falciparum* in the surrounding countries (Figure 1), a situation analogous to that described for Thailand before control efforts reduced the prevalence of this parasite to its current levels. There is a recent report that suggests that *P. vivax* is gradually becoming the predominant species in Cambodia, a shift not associated with resistance to chloroquine [52]. It would be interesting to define the factors that are implicated in such alterations in parasite species distribution, an aim that would require detailed longitudinal epidemiological observations.

Thailand thus offers a unique opportunity to study two malaria parasites that exist side-by-side in residents of the endemic regions. An assessment of independent data gathered by researchers and clinicians working in Thailand raises several fundamental questions about the epidemiology and biology of malaria, and offers a glimpse into the potential consequences of mixed species infections.

The tantalizing clinical and parasitological associations that are emerging from epidemiological observations gain particular relevance with the realization that mixed infections are inordinately prevalent.

Beneficial associations

The Shoklo camp on the Thai–Myanmar border in western Thailand, where Karen populations displaced by armed conflict took refuge since 1984, has provided an excellent setting for detailed long-term epidemiological and clinical observations of malaria. However, malaria epidemiology at this site and at that time is unlikely to be representative of the rest of Thailand, and patient recruitment in the early 1990s was coincident with the peak crisis of mefloquine resistance and the first efforts to introduce the combination of artemisinin and its derivative with other anti-malarial drugs. In a preliminary study conducted in 1991–1992, *P. falciparum* and *P. vivax* each accounted for 37% and 53%, respectively, of infections observed, a situation reflected in other endemic areas. The overall prevalence rate for *P. falciparum* did not exceed 4% and for *P. vivax* 6%, and examination by microscopy revealed that mixed infections occurred in ~10% of the parasitemic episodes recorded [53]. This level of mixed infections was confirmed in a subsequent extensive study of malaria in the camp residents over a period of two years (1990–1992). The first indication of an influence of mixed infections was obtained when it was calculated that the probability that patients presenting with acute *P. falciparum* infections will develop severe disease or complications was reduced fourfold in patients where *P. vivax* was also present [54]. Another interesting benefit conferred by mixed infections was noted in a study of artesunate/mefloquine treatment efficacy conducted in the same population between 1992 and 1995. In this study, where mixed species infections (*P. falciparum* and *P. vivax*) were observed in 23% of the patients recruited, the number of treatment failures for *P. falciparum* in the group where this parasite was found alone was double that observed in the group where the two parasites were present simultaneously [55]. Further clinical differences between patients infected with *P. falciparum* alone or with both parasite species emerged in a study of anemia [56]. The simultaneous presence of *P. vivax* on admission was not only associated with an attenuation of anemia, but also with a reduced risk of developing anemia during follow-up.

The evidence for consequent interactions between the parasite species extended beyond clinical associations. A curious effect of mixed infections was brought to light in a study of the genetic diversity of *P. falciparum* infections in individuals at Shoklo [57]. Coincident *P. vivax* infections were significantly associated with a lower genetic complexity of *P. falciparum* infections [57]. The prevalence and extent of mixed genotypes in individual infections are somewhat linked to the dynamics of malaria transmission, which in turn influence the pattern of morbidity and the spread of drug-resistant parasites. The relevance of the above observations was reinforced by results from a study of *P. falciparum* gametocyte carriage in Shoklo [58]. The presence and numbers of *P. falciparum* gametocytes (those of *P. vivax* were not recorded) were noted in slides obtained

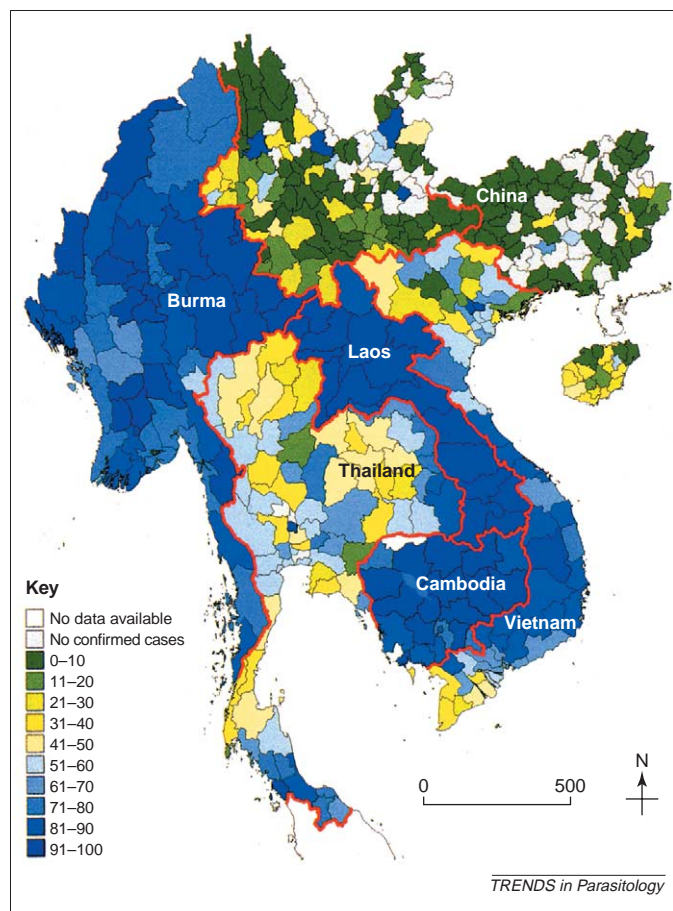


Figure 1. The proportion of *Plasmodium falciparum* and *Plasmodium vivax* cases reported by the malaria programs of the health ministries of Cambodia, China, Lao PDR, Vietnam, Myanmar and Thailand. Although data collection and analysis differed between countries, the diagram broadly reflects the general pattern of species distribution. Scale bar = 500 km. Reproduced, with permission, from Ref. [77].

on admission and during follow-up. Patients with mixed species infections were four-times less likely to present with patent *P. falciparum* gametocytemia when compared with pure *P. falciparum* infections. Furthermore, the density of these gametocytes tended to be lower in the mixed infection group of patients. Furthermore, when *P. vivax* was also found mixed with *P. falciparum* in the admission, the likelihood of observing *P. falciparum* gametocytes during treatment follow-up (4–9 weeks) was decreased threefold as compared with that in patients with a pure *P. falciparum* infection on admission. However, it is not clear that such changes will lead to reduced transmission. In *Aotus* monkeys, an increase in the infectivity to mosquitoes was observed in reciprocal, sequential, experimental infections with *P. falciparum* and *P. vivax* as compared with single infections [59]. Conversely, it was found that *P. falciparum* gametocyte production is enhanced by prior or concurrent *P. malariae* infection, although only a minor decrease of *P. malariae* gametocyte production is obtained by similar *P. falciparum* infections [15,16]. Clearly, further studies are required.

Too many mixed infections

The levels of mixed infections recorded in Shoklo contrast sharply with those recorded for the rest of the country. Indeed, *P. falciparum* and *P. vivax* were found together only in 0.3–0.7% of the total positive blood smears recorded in Thailand [51]. If this were the true prevalence of mixed species infections, then any interactions between the species would have minimal impact on public health. However, there are compelling indications that the higher levels of mixed infection recorded in Shoklo might actually be typical across the endemic areas of Thailand.

The first evidence that a higher incidence of double infections might occur in Thailand was provided by a series of *in vivo* drug efficacy studies associated with a long-term follow-up of 1–2 months at the Bangkok Hospital for Tropical Diseases, a setting where malaria transmission does not occur. The remarkable observation, initially made in 1987, that *P. vivax* appeared in the blood of a third of the patients treated for *P. falciparum* [60] also held for numerous subsequent studies in Thailand and in other countries [45]. Similar observations of frequent *P. vivax* relapses were also made in Brazil [61]. Furthermore, it was also found that ~10% of patients treated for *P. vivax* presented with *P. falciparum* infections during follow-up [62–66]. Further evidence was provided by PCR detection studies. Analysis of admission samples collected in Bangkok [67,68] and in malaria clinics throughout the country [34,35,68,69] amply confirmed that mixed infections are common. Incidentally, these studies also demonstrated that failure to detect cryptic *P. vivax* on admission was not solely due to the presence of dormant stage in the liver (the hypnozoite).

Taken together, these observations indicate that between a third to a half of the malaria infections recorded in Thailand are mixed species infections. Such a high level, noteworthy in itself, is baffling when placed in the country's epidemiological context. Malaria in Thailand has long been confined to a few provinces bordering

neighboring countries, mainly Myanmar and Cambodia, following the implementation of highly successful control programs initiated in the 1950s. The annual parasite incidence (API, expressed as the number of cases per 1000 population from passive and active case detection) of around 2.0 is relatively low, although asymptomatic infections and cases treated in private clinics are poorly recorded. Infections by both parasite species, which occur mainly in non-immune residents or visitors, usually lead to clinical symptoms. An extensive, well-organized network of malaria clinics ensures that most cases are recorded and treated appropriately. The bulk of transmission is due to mosquitoes belonging to three species complexes (*Anopheles dirus*, *Anopheles minimus* and *Anopheles maculatus*). Crucially, transmission intensities are estimated to be low, with an average effective inoculation rate (EIR) of one infective bite per person per year, a figure that apparently rarely varies geographically or temporally by more than one order of magnitude. However, it should be noted that measurement of EIR is fraught with difficulties.

The perceived low transmission intensities (as compared with >100 infective bites per person per year in many hyperendemic African regions) and the adequate coverage and treatment of generally non-immune patients are not consistent with the demonstrated levels of mixed species infections. Thus, the equal prevalence of *P. falciparum* and *P. vivax* might contribute, but cannot account for these levels.

Mixed infections can be acquired either simultaneously or by sequential infections. We define 'simultaneous inoculation' as occurring either through the same infective bite or through two consecutive bites in quick succession, within one week of each other. If this were the dominant route to mixed infections in Thailand, then one of two conclusions applies. First, the EIR levels are grossly underestimated, and the infected mosquitoes are equally likely to harbor one or the other of the two parasite species. High EIR values are unlikely given the relatively low prevalence of clinical cases in the broadly non-immune residents. Second, if, by contrast, persons are only truly subjected to very few infective bites each year, then a substantial proportion of infected mosquitoes must harbor both *P. falciparum*- and *P. vivax*-infective sporozoites in their salivary glands. The fact that 30 of the 113 patients who developed patent *P. vivax* infections following treatment of *P. falciparum* had no previous history of malaria and had acquired their infection by a single visit to an endemic region [60], lends some credence to this scenario. Individual inoculations of polyclonal *P. falciparum* sporozoites were also consistent with genetic studies of this parasite in Shoklo [57]. Irrespective of the EIR, a demonstration of a simultaneous acquisition of mixed infections would provide evidence that, in primary infections, either species of parasites can suppress the multiplication of the other, or its appearance in the peripheral circulation.

Alternatively, mixed infections might arise as a result of sequential inoculations, separated by a long period of time. If this were the case for the majority of mixed *P. falciparum*/*P. vivax* infections in Thailand, it would

imply that treatment regimes, although clinically effective, often fail to eradicate the parasite. This is consistent with the high prevalence of drug-resistant *P. falciparum* parasites in Thailand, for which optimal treatment is only obtained by combination therapies containing an artemisinin derivative. Nonetheless, unexplained treatment failures of demonstrably drug-susceptible parasites do occur [70]. This might indicate that latent merozoites, the merophore described in rodent malaria [71], might also be found in *Plasmodium* species of humans. For *P. vivax*, lengthy persistence of the infection is probably mainly due to the hypnozoites, which for Thai strains cause relapses at three-weekly intervals [65,72]. Although primaquine is part of the recommended treatment, patients often fail to comply with the 14-day treatment, and those with glucose-6-phosphate deficiency predisposing to hemolytic anemia (a sizeable proportion of patients in Thailand) are not treated with primaquine. The reappearance of *P. falciparum* after clearance of *P. vivax* by chloroquine, a drug to which Thai *P. falciparum* parasites are not susceptible, implies that a *P. vivax* episode depresses the mechanisms that maintain *P. falciparum* as chronic. By contrast, treatment of *P. falciparum* is achieved with drugs to which *P. vivax* blood stages are susceptible; thus, the subsequent appearance of *P. vivax* implies that a *P. falciparum* episode reactivates *P. vivax* hypnozoites. Irrespective of the reactivation mechanism, mixed infections owing to sequential infections would require a high proportion of the population to be asymptomatic carriers. This proposition can be tested relatively easily for *P. falciparum* where hypnozoites are not known to occur.

Mixed perspectives

Two immediate conclusions can be drawn from the observations made in Thailand and elsewhere: (i) mixed-species malaria infections are common; and (ii) they are likely to have an impact on the morbidity and epidemiology of malaria. The growing trend to include non-*P. falciparum* infections in the analysis of epidemiological and clinical data is providing glimpses of interesting associations with mixed infections in other areas where the different species are prevalent such as Papua New Guinea [73], or in Africa where *P. falciparum* dominates the clinical picture [74]. However, this aspect of malaria is still in its infancy and insights into underlying mechanisms remain in the realm of speculation.

In future investigations, the definition of mixed infections must be broadened to encompass sequential parasitemic episodes. The data from cross-sectional surveys and those based on results from a single blood sample per person are inadequate. Thus, pre-requisites for investigation of mixed infections include (i) longitudinal studies using adequate sampling intervals consistent with the natural course of infection by the different species, (ii) an accurate method of parasite detection, and (iii) robust and validated statistical tests. Although the limitations of microscopy can be minimized by judicious longitudinal observations, such as those made in Thailand, current PCR-based methodologies offer the most suitable alternative, and quantitative PCR protocols would be the ideal tool to employ.

Important indications of the influence of mixed infections in humans can now only be obtained from detailed longitudinal observations, but investigations of the mechanisms through which parasites interact would necessitate detailed experimental observations that are ethically excluded for humans. *Plasmodium* infections in rodent models, by no means a satisfactory substitute, nonetheless provide the only practical laboratory mammalian model to identify biological, immunological and pathological phenomena that are implicated. Relevance to human infections can then be ascertained by suitable prospective field observations.

The relevance of interactions between the different *Plasmodium* species extends beyond mere academic curiosity. Observations in Thailand and Vanuatu sustain the hypothesis, considered as far-fetched by some, that *P. vivax* somewhat 'vaccinates' against the clinical severity of *P. falciparum*. A similar influence might be exerted by the cryptic, but nonetheless prevalent, *P. ovale* and *P. malariae* in Africa. It would then be legitimate to wonder whether suppression of the 'minor' species would lead to an increase in morbidity. This scenario is consistent with the increase of mortality resulting from the continued use of chloroquine as a first line treatment in Africa in the face of mounting resistance to this drug by *P. falciparum* [75,76], but not by the other parasite species. Whether implementation of control measures specific to *P. falciparum*, for example the long-awaited vaccine, will alter the epidemiology of the remaining species remains to be determined.

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