An observational study of malaria in British travellers: *Plasmodium ovale wallikeri* and *Plasmodium ovale curtisi* differ significantly in the duration of latency

Debbie Nolder,[1] Mary C Oguike,[2] Hector Maxwell-Scott,[2,3] Hatoon A Niyazi,[2] Valerie Smith,[1] Peter L Chiodini,[1,3] Colin J Sutherland[1,2,3]

For numbered affiliations see end of article.

Correspondence to
Dr Colin J Sutherland;
colin.sutherland@lshtm.ac.uk

ABSTRACT

Objectives: Ovale malaria is caused by two closely related species of protozoan parasite: *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* Although clearly distinct genetically, there have been no studies comparing the morphology, life cycle or epidemiology of these parasites. We tested the hypothesis that the two species differ in the duration of latency prior to presentation with symptoms of blood-stage infection.

Design: PCR was used to identify *P ovale curtisi* and *P ovale wallikeri* infections among archived blood from UK malaria patients. Latency periods, estimated as the time between entry into the UK and diagnosis of malaria, were compared between the two groups.

Setting: UK National Reference Laboratory.

Participants: None. Archived parasite material and surveillance data for 74 *P ovale curtisi* and 60 *P ovale wallikeri* infections were analysed. Additional epidemiological data were taken from a database of 1045 imported cases.

Outcomes: None.

Results: No differences between the two species were identified by a detailed comparison of parasite morphology (N=9, N=8, respectively) and sex ratio (N=5, N=4) in archived blood films. The geometric mean latency period in *P ovale wallikeri* was 40.6 days (95% CI 28.9 to 57.0), whereas that for *P ovale curtisi* was more than twice as long at 85.7 days (95% CI 66.1 to 111.1; p=0.002). Further, the proportion of ovale malaria *sensu lato* which occurred in patients reporting chemoprophylaxis use was higher than for *Plasmodium falciparum* (OR 7.56; p<0.0001) or *P vivax* (OR 1.82; p<0.0001).

Conclusions: These findings provide the first difference of epidemiological significance observed between the two parasites which cause ovale malaria, and suggest that control measures aimed at *P falciparum* may not be adequate for reducing the burden of malaria caused by *P ovale curtisi* and *P ovale wallikeri*.

ARTICLE SUMMARY

Article focus
- We hypothesised that the duration of latency may differ between the two *Plasmodium* species that cause ovale malaria.

Key messages
- On average, *Plasmodium ovale curtisi* displayed a latency period more than twice as long as *P ovale wallikeri* in imported cases in the UK.
- Both species were found in patients that reported chemoprophylaxis use

Limitations of this study
- Reference-level samples are prone to selection bias.
- The reliability of self-reported data on prophylaxis use is low.

INTRODUCTION

Malaria is a mosquito-borne disease caused in humans by six protozoan parasites *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium knowlesi*, *Plasmodium malariae*, *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri*. The two latter species were only recently differentiated, and are estimated to cause more than 15 million African cases of ovale malaria each year.[1] *P ovale curtisi* and *P ovale wallikeri* are thought to have diverged from each other 1–2 million years ago, probably owing to the independent introductions of proto-ovale parasites from primitive primates into the early hominid lineage.[2] The two species are found together across their range in Africa and Asia, sometimes coinfecting a single individual,[1 3–7] providing compelling evidence that true biological separation, and not geographic isolation (allopatry), has prevented genetic recombination between

P ovale curtisi and *P ovale wallikeri*. However, to date, no differences in morphology, life cycle, epidemiology or clinical presentation in symptomatic patients have been reported.

P ovale curtisi and *P ovale wallikeri* are both thought to form latent hypnozoite stages in the liver, similar to those of *P vivax* or the simian parasite *Plasmodium cynomolgi*.[8] This has long been considered a general feature of ovale malaria.[9] Activation of hypnozoites may thus initiate blood-stage parasitaemia long after exposure to biting mosquitoes; this can also lead to a secondary febrile attack (relapse) some time after the chemotherapeutic cure of a primary illness.[10] Hypnozoites are insensitive to almost all antimalarial drugs and therefore can delay the onset of clinical malaria in people such as travellers protected from a primary malaria episode by temporary chemoprophylaxis; even in the absence of antimalarials, hypnozoites of *P vivax* are implicated in the observed delay in the occurrence of primary malaria episodes in this species.[11] This latency period can present considerable difficulties for diagnosis of imported malaria cases in non-endemic countries, as the presenting symptoms appear to be unlinked to recent foreign travel.[12–14] It has been suggested that variation in the duration of hypnozoite dormancy, and in the periodic patterns of relapse, could lead to differences in the measured latency period between *P ovale curtisi* and *P ovale wallikeri*,[1] but there are as yet no published data which support this hypothesis. Further, a recent appraisal of the relevant literature has led Richter *et al*[15] to question the evidence for the existence of hypnozoites in ovale malaria at all. There is, therefore, a need for further study of relapses in ovale malaria; one convenient approach is to compare the latency periods in travellers infected with *P ovale curtisi* or *P oval wallikeri*, and to evaluate the effect, if any, of chemoprophylaxis in lengthening the observed latency.

We analysed cases of imported ovale malaria referred to the Health Protection Agency Malaria Reference Laboratory (HPA MRL) for which both the date of arrival in the UK and date of diagnosis were available, and for which an aliquot of the original blood sample had been retained, permitting confirmation of the presence of *P ovale curtisi* or *P ovale wallikeri* in each case by PCR. Parasite morphology data were collected by re-examination of the original blood films for a small subset of samples; travel histories, demographic data and records of chemoprophylaxis use were sourced from the MRL malaria surveillance database. The duration of latency was estimated for each case, and this was compared between the two species groups.

METHODS
Collection of data and samples
The UK HPA MRL maintains an electronic database of cases of malaria that have occurred in the UK since 1987[16]; ad hoc recapture analysis suggests that approximately 56% of imported malaria cases are represented in the database.[17] This surveillance function is based on passive case reporting by clinicians using data collection methodologies that have not changed over the last 20 years,[18] although since 2005 all service users sending blood films for examination are requested to also send an aliquot of the diagnostic blood sample to the MRL for PCR investigations.[19] We identified cases of ovale malaria notified to the MRL between January 2003 and August 2011 with sufficient data to estimate the latency period prior to diagnosis, and for which a blood sample had been provided for DNA extraction.

Ethics statement
This work was undertaken as part of the surveillance activity of the MRL, as previously described.[18] All patient-identifiers were removed from the data prior to extraction from the HPA MRL database. No additional blood samples were taken from patients, other than those required for the primary diagnosis of malaria.

Microscopic diagnosis
The parasite species present in all isolates were identified by examination of Field's-stained thick smears and Giemsa-stained thin smears under oil immersion at 500–1000× magnification. Where the morphology was not conclusive, species designations were confirmed by PCR as described below. Male gametocytes of *P ovale* spp. were distinguished from female gametocytes by the presence of a larger nucleus and more scattered haemozoin (dark pigment).

Plasmodium species identification by PCR
Genomic DNA was extracted from blood samples using the Qiagen blood mini kit (Qiagen, UK), according to the manufacturer's instructions. Parasite species were distinguished by PCR amplification of small subunit ribosomal RNA genes, using minor modifications of methods published elsewhere,[20–22] to ensure amplification, but not discrimination, of both *P ovale curtisi* and *P ovale wallikeri*.[3 7] Discrimination of *P ovale curtisi* from *P ovale wallikeri* was carried out by conventional amplification of the *potra* locus and quantitative real-time PCR (qPCR) amplification of the *porbp2* locus, as previously described.[3]

Statistical analysis
The latency period, in days, for each episode of malaria was calculated by subtracting the date of return from travel (the last possible point in time when parasites could have been introduced by an infectious *Anopheles* bite) from the date of diagnosis in the UK.

Continuous variables were compared between species using geometric means, and statistical significance was evaluated by the Wilcoxon rank-sum test. Associations between pairs of binary variables were investigated in 2×2 contingency tables, and statistical significance was tested using the χ^2 distribution, with Fisher's exact correction when the expected frequency in any cell was

5 or less. All statistical analyses were performed in STATA v.12 (Timberlake, College Station, Texas, USA).

RESULTS

Seven hundred and fifty-seven cases of malaria caused by *P ovale* spp. were reported to the HPA MRL from November 2003 (when EDTA-blood sample archiving in the MRL was started) until the end of August 2011, for which 573 (75.7%) blood samples, originally submitted to the laboratory for primary or confirmatory diagnosis, were available for this study. Among these, we identified 134 samples for which sufficient details of travel history were available to estimate the latency period.

Species discrimination at the *potra* and *porbp2* loci for all 134 isolates identified 74 (55.2%) as *P ovale curtisi* and 60 (44.78%) as *P ovale wallikeri*. Species assignments by the two methods were in complete agreement for each isolate. No mixed infections were identified in which both species were present.

Parasite morphology and sex ratio

A small sample of 17 *Povale* spp. infections from 2011 and 2012, including two isolates from the 134 in the main analysis, were selected on the basis that good-quality blood films (both thick and thin smears) showing multiple life cycle stages were available, with an estimated parasitaemia of at least 5 parasitised cells per 10 000 erythrocytes (0.05%; range 0.05–1.2%). These comprised nine *P ovale curtisi* and eight *P ovale wallikeri* infections. A single microscopist, blinded to the species designations, collected descriptive data on each, including the number of merozoites in each schizont-stage parasite, the proportion of enlarged cells and the arrangement and appearance of pigment (haemozoin) depositions in the parasite cytoplasm. These data were checked and confirmed by another team member before unblinding. None of the morphological parameters differed significantly between the two species in this small sample (data not shown). The sex ratio (proportion of all gametocytes that were male) was determined for nine isolates in which at least 20 gametocytes (range 24–117) were identified. The observed sex ratio for both species was similar: for *P ovale curtisi*, the geometric mean sex ratio was 0.33; 95% CI 0.26 to 0.40 (N=5) and for *P ovale wallikeri*, the sex ratio was 0.27, 95% CI 0.19 to 0.39 (N=4; p=0.32).

Patients, travel histories and prophylaxis use

Assuming that each infection was acquired during the most recently reported journey, patients contracted ovale malaria in at least 15 African countries, with Nigeria and Ghana being the most commonly identified places of origin, reflecting the popularity of these destinations in travellers returning to or visiting the UK (table 1). Stratifying the samples by geographic origin as 103 from West Africa (including Cameroon) and 28 from East/Southern Africa (including the Congo), no

Table 1 Recent travel destinations of patients contributing parasite isolates

	Plasmodium ovale curtisi	Plasmodium ovale wallikeri
Burundi		1
Cameroon	2	7
Congo	1	
Ghana	13	9
Guinea	1	
Ivory coast	1	2
Kenya	1	
Madagascar		1
Malawi	2	2
Mozambique	1	
Nigeria	33	24
Sierra Leone	5	6
Tanzania	1	
Uganda	7	5
Zambia		1
Africa overland	1	
East Africa		2
Southern Africa	1	
	74	60

difference was found in the proportion of the two species in these two strata: *P ovale curtisi* comprised 53.4% and 57.1%, respectively (p=0.72). The three remaining isolates were lacking information on specific destinations within Africa.

Age, gender, prophylaxis use and duration of travel of the study sample, stratified by parasite species, are presented in table 2. There were no significant associations between parasite species and the age (p=0.16) or sex (p=0.509) of the patient, nor with the reported duration of travel (p=0.42).

Although the MRL does not systematically collect data on the use of mosquito protection measures such as repellents or bednets, information on malaria chemoprophylaxis use is collected and was available for 112 patients. Overall, 37 (33%) patients with ovale malaria reported the use of some form of chemoprophylaxis (table 2), but this did not significantly affect which species of parasite was present: of those infected with *P ovale curtisi*, 21 used prophylaxis, whereas of those infected with *P ovale wallikeri*, 16 used prophylaxis (p=0.46). Among the different drugs used for prophylaxis, atovaquone-proguanil was more often used by those who later presented with an infection of *P ovale wallikeri*, but this was not statistically significant (OR 3.6, 95% CI 0.59 to 26.3; Fisher's exact p=0.14).

To compare these data with the effect of prophylaxis use on other *Plasmodium* species, both non-relapsing and relapsing, we extracted information on prophylaxis use among UK malaria patients between 1991 and 2010, including a sample (evenly spread across the time period) of those presenting with *P falciparum* (N=2814; 7.5% reported at least some prophylaxis use) and all

Table 2 Characteristics of the study population

	Plasmodium ovale curtisi 74 (55.2%)	*Plasmodium ovale wallikeri* 60 (44.8%)
Age (years) mean	34.8	31.5
(95% CI; range)	(31.7 to 37.9; 12–71)	(27.9 to 35.1; 2–62)
Gender		
Females, N (proportion)	21 (28.4%)	14 (23.3%)
Prophylaxis use*		
Any (proportion; N)	21 (36.2%; 58)	16 (29.6%; 54)
Mefloquine	10	3
Doxycycline	6	5
Atovaquone-proguanil	3	6
Duration of travel (days)*		
Geometric mean (95% CI)	43.1 (30.6 to 60.7)	52.6 (37.3 to 74.0)
N	39	36

*Data were incomplete for these categories.

available data for *P vivax* (N=4,133; 23.7% used prophylaxis). These data were compared with all available data for *P ovale* spp. (N=1,045; 36.2% used prophylaxis). Thus, the proportion of malaria cases caused by *P ovale* spp., which occurred among people reporting prophylaxis use, was higher than the corresponding figure for *P falciparum* (OR 7.56; 95% CI 6.23 to 9.17; p<0.0001), and also significantly higher than the figure for the known relapsing parasite *P vivax* (OR 1.82; 95% CI 1.57 to 2.11; p<0.0001). However, this latter comparison may be affected by the fact that chemoprophylaxis is less frequently recommended for travellers to Pakistan and India, from where the majority of cases of vivax malaria originated, than for Africa, the origin of virtually all ovale malaria cases in the UK.

Latency analysis
The geometric mean latency period for *P ovale curtisi* infections was 85.7 days (95% CI 66.1 to 111.1) and for *P ovale wallikeri* infections was 40.6 days (95% CI 28.9 to 57.0; figure 1), giving a geometric mean difference in latency period for these species of 45.1 days (Wilcoxon rank-sum: z=3.087; p=0.0020). These compare with an estimated geometric mean latency for *P vivax*, from the MRL database, of 57.2 days (95% CI 54.8 to 59.8 days; N=4,920). For each of these relapsing species, no attempt was made in the analysis to distinguish primary infections from relapse infections—all cases of each species with sufficient data were analysed together. One *P ovale curtisi* infection displayed an extraordinary latency period of 1083 days; removal of this outlier only slightly reduced the estimate of the geometric mean latency period for this species to 82.7 days (95% CI 64.2 to 106.6), and the interspecies difference remained significant (Wilcoxon rank-sum: z=2.984; p=0.0028).

Information on prophylaxis use was available for 112 cases, of whom 37 reported taking at least some chemoprophylaxis. Of the 58 evaluable cases with *P ovale curtisi* infection, geometric mean latency was 88.6 days (95% CI 59.0 to 132.8) among the 37 who reported taking no prophylaxis, compared with 69.9 days (95% CI 45.2 to 108.2) among the 21 reporting at least some prophylaxis use (p=0.544). Of the 54 evaluable cases with *P ovale wallikeri*, geometric mean latency was 33.9 days (95% CI 21.9 to 52.6) among the 38 who did not report prophylaxis use. However, the corresponding geometric mean latency was almost twice as long among the 16 using prophylaxis at 60.4 days (95% CI 33.7 to 108.1; p=0.179), suggesting a weak, non-significant impact of chemoprophylaxis on the latency period of *P ovale wallikeri*.

DISCUSSION
Latency periods differ for *P ovale curtisi* and *P ovale wallikeri*
In this descriptive study, the human malaria parasites *P ovale curtisi* and *P ovale wallikeri* are shown to differ
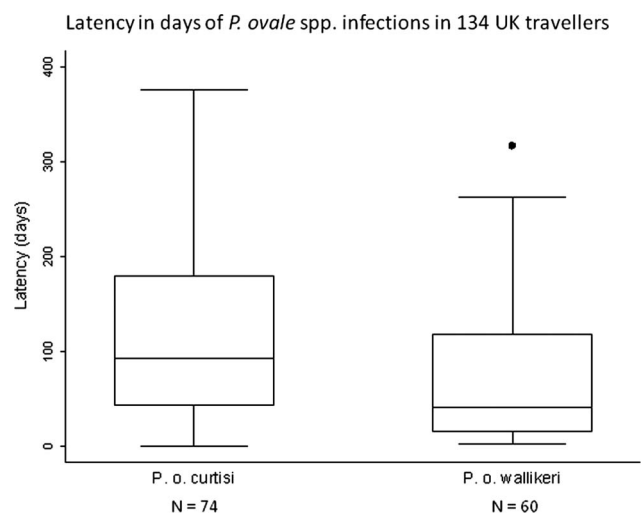

Figure 1 Latency in days of *Plasmodium ovale* spp. infections in 134 UK travellers. The geometric mean number of days elapsing between arrival in the UK and diagnosis of ovale malaria is given for all patients. The midline of each box-plot is the median, with the edges of the box representing the interquartile interval. Whiskers delineate the 5th and 95th percentiles.

significantly in the latency period in travellers with ovale malaria diagnosed in the UK. Despite substantial and compelling genetic divergence between the two species,[1] [3] no differences have hitherto been found in morphology, geographic distribution, epidemiology or clinical features of disease. The current analysis thus provides the first evidence that malaria infections caused by these two similar but genetically distinct species differ in a clinically important feature. Our data also suggest that ovale malaria, caused by either *P ovale curtisi* or *P ovale wallikeri*, is less likely to be prevented by chemoprophylaxis than are the other forms of human malaria. Thus, these two species together present particular challenges to malaria control and eradication programmes.

Long latency periods and the delayed onset of symptomatic malaria present considerable difficulties for disease diagnosis and the swift provision of appropriate treatment.[13] Delayed diagnosis can worsen clinical outcomes and increase morbidity,[23] thus increasing the costs to healthcare systems. Our assessment of latency periods in imported cases of ovale and vivax malaria demonstrates that delayed onset malaria must remain a differential diagnosis of fever when any history of travel to malaria-endemic countries is months, or even years, in the past. The longest recorded period of latency in our dataset was 1083 days, in a case of *P ovale curtisi* infection.

P. ovale spp. and relapse malaria

Richter *et al*[15] have argued that insufficient evidence is available to unequivocally demonstrate that *P ovale* spp. are able to form 'true' hypnozoites which subsequently cause relapse malaria. Although lack of biological evidence from ex vivo liver-stage studies is indeed a problem, the data presented here would support the presence of ovale hypnozoites in two ways. First, a relatively high proportion (36.2%) of imported cases of ovale malaria are in people who report the use of prophylaxis, being much higher than the proportion in those who contracted falciparum malaria (6.4%) and slightly higher than for vivax malaria (23.7%), a known relapsing malaria species with well-described hypnozoites. Second, the observed latency periods for the two ovale malaria species, at 85.7 and 40.6 days, respectively, are similar to those for vivax malaria cases (57.2 days). These findings are consistent with both *P ovale curtisi* and *P ovale wallikeri* being true relapse malaria species, and indirectly infer the presence of hypnozoites in both species. Further biological studies of *P ovale* spp., perhaps in cultured liver cells, are required to provide a further understanding of latency in ovale malaria.

Species differentiation/morphology of *P ovale curtisi* and *P ovale wallikeri*

Our morphological observations did not identify any feature that could discriminate between *P ovale curtisi* and *P ovale wallikeri*, but continuing careful descriptive microscopy of these two parasites may eventually find such a criterion. We present here the first attempt to compare the sex ratio of the two species; although no difference was found in this small sample, the estimated sex ratio of approximately 0.3 (ie, just over 2 male gametocytes to every female gametocyte in peripheral blood smears) is quite different from the estimate of 0.13 (ie, eight males for every female gametocyte) observed with cultured *P falciparum*.[24] Further work is required to confirm this estimate, and studies of gamete emergence (ex-flagellation) in ex vivo preparations of the gametocytes of *P ovale* spp. may also shed further light on the true sex ratio.

Study limitations

There are several limitations to our study. First, as a reference level laboratory receiving parasite-infected blood from a wide variety of source facilities across the UK, the available material does not come as the result of carefully planned sampling strategies, but simply reflects the most common malaria-endemic destinations for UK travellers; the most common countries of origin of African migrants to the UK; and laboratory referral patterns. Although this does include West Africa and East/Southern Africa, all of Asia and many countries in Africa are missing from our sample set. For example, French workers have recently described 90 imported ovale malaria cases of both species from the Ivory Coast and the Comoros Islands,[6] locations contributing only three individuals in our sample set (table 1). Second, in the absence of an exact date of infection, our measure of latency uses the date of arrival in the UK as a proxy. This is an obvious source of imprecision, but means that in every case the latency period used in the analysis is a minimum estimate. Further, this imprecision is expected to be of approximately equal magnitude, on average, for infections with *P ovale curtisi* as for those with *P ovale wallikeri*. Therefore, there is no a priori reason to consider this as a source of confounding. Third, we do not currently have the tools to distinguish primary ovale malaria attacks, being the first blood stage parasite expansion since mosquito inoculation, from relapse malaria caused by the activation of previously dormant hypnozoites; in some cases, these may have been present for years. Therefore, our latency data for each species may mask two distinct natural histories; future studies with larger datasets could benefit from model-fitting to explore this aspect in more detail. Finally, the data used to determine prior prophylaxis use is notoriously unreliable: patients may have taken incomplete or sporadic courses which did not provide sufficient chemo-protection.[25] Nevertheless, these data will be equally flawed across all the species used for the comparative analysis, and thus the broad differences we observed are likely to be biologically real. Interestingly, there is a hint in our data that prophylaxis affects the latency period in *P ovale wallikeri* infections more than in *P ovale curtisi* infections. Further studies, deploying more detailed patient histories, are needed to investigate this further.

Recommendations

Delayed onset malaria is responsible for a small but important subset of imported malaria cases in the UK, and *P. ovale* spp. parasites are disproportionate contributors to this. Longitudinal studies in endemic countries are needed to investigate the mechanisms of these apparent prophylaxis breakthroughs. *P ovale curtisi* and *P ovale wallikeri* differ in latency patterns; further studies of the clinical and epidemiological features of these two parasites may identify other differences.

Author affiliations
[1]Public Health England (formerly HPA), Malaria Reference Laboratory, Faculty of Infectious & Tropical Diseases, London School of Hygiene & Tropical Medicine, London, UK
[2]Department of Immunology & Infection, Faculty of Infectious & Tropical Diseases, London School of Hygiene & Tropical Medicine, London, UK
[3]Department of Clinical Parasitology, Hospital for Tropical Diseases, University College London Hospitals Trust, London, UK

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