RESEARCH ARTICLE

Plasmodium vivax and *Plasmodium falciparum* at the Crossroads of Exchange among Islands in Vanuatu: Implications for Malaria Elimination Strategies

Chim W. Chan[1]*, Naoko Sakihama[2¤a], Shin-Ichiro Tachibana[2¤b], Zulkarnain Md Idris[1], J. Koji Lum[3,4,5], Kazuyuki Tanabe[2†], Akira Kaneko[1,6,7]

1 Island Malaria Group, Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden, **2** Laboratory of Malariology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan, **3** Laboratory of Evolutionary Anthropology and Health, Binghamton University, Binghamton, New York, United States of America, **4** Department of Anthropology, Binghamton University, Binghamton, New York, United States of America, **5** Department of Biological Sciences, Binghamton University, Binghamton, New York, United States of America, **6** Department of Parasitology, Graduate School of Medicine, Osaka City University, Osaka, Japan, **7** Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan

† Deceased.
¤a Current address: Laboratory of Biology, Osaka Institute of Technology, Ohmiya, Asahi-ku, Osaka, Japan
¤b Current address: Department of Biology and Geosciences, Graduate School of Science, Osaka City University, Osaka, Japan
* chim.chan@ki.se

OPEN ACCESS

Citation: Chan CW, Sakihama N, Tachibana SI, Idris ZM, Lum JK, Tanabe K, et al. (2015) *Plasmodium vivax* and *Plasmodium falciparum* at the Crossroads of Exchange among Islands in Vanuatu: Implications for Malaria Elimination Strategies. PLoS ONE 10(3): e0119475. doi:10.1371/journal.pone.0119475

Academic Editor: Georges Snounou, Université Pierre et Marie Curie, FRANCE

Received: September 1, 2014

Accepted: January 20, 2015

Published: March 20, 2015

Copyright: © 2015 Chan et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: DNA sequences described in this paper are available in the GenBank database (accession numbers AB116596-AB116607, AB539022-AB539045, and AB539540-AB539553). Frequencies of haplotypes are contained within the paper and its supporting information files.

Funding: Financial support for this study was provided to AK by the Swedish Research Council (www.vr.se, grant numbers 523-2009-3233, 348-2012-6346, and 348-2013-6311), the Japan Society for the Promotion of Science (JSPS, https://www.jsps.go.jp/english/) Core-to-Core Program, B. Asia-Africa

Abstract

Understanding the transmission and movement of *Plasmodium* parasites is crucial for malaria elimination and prevention of resurgence. Located at the limit of malaria transmission in the Pacific, Vanuatu is an ideal candidate for elimination programs due to low endemicity and the isolated nature of its island setting. We analyzed the variation in the merozoite surface protein 1 (*msp1*) and the circumsporozoite protein (*csp*) of *P. falciparum* and *P. vivax* populations to examine the patterns of gene flow and population structures among seven sites on five islands in Vanuatu. Genetic diversity was in general higher in *P. vivax* than *P. falciparum* from the same site. In *P. vivax*, high genetic diversity was likely maintained by greater extent of gene flow among sites and among islands. Consistent with the different patterns of gene flow, the proportion of genetic variance found among islands was substantially higher in *P. falciparum* (28.81–31.23%) than in *P. vivax* (-0.53–3.99%). Our data suggest that the current island-by-island malaria elimination strategy in Vanuatu, while adequate for *P. falciparum* elimination, might need to be complemented with more centrally integrated measures to control *P. vivax* movement across islands.

Science Platforms, JSPS KAKENHI (grant numbers 24390141 and 26257504), Health Labour Sciences Research Grant, Research on Global Health issues, and Nagasaki University Institute of Tropical Medicine collaborative research grant. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Renewed commitment to control malaria over the last decade has resulted in major reductions in case incidence and disease mortality rates, and 32 of 99 countries with endemic malaria are pursuing an elimination strategy [1,2]. Outside of sub-Saharan Africa, *Plasmodium vivax* infections present unique and additional challenges for elimination due to the parasite's propensity to relapse and the limitations of primaquine [1,3]. Further, malaria resurgence has the potential to undermine control and elimination efforts [4–6]. To this end, parasite population genetics studies are fundamental in identifying routes of transmission and gene flow, such that appropriate strategy for control and intervention might be implemented [7].

Islands provide an ideal model for natural ecological experiments and present a great opportunity for intervention studies. Vanuatu is an archipelago consisting of 68 inhabited islands located at the southeastern limit of malaria transmission in the Pacific. Malaria is mainly hypo- to meso-endemic, with a general decrease in annual parasite incidence (API) from the northwest to the southeast. *P. falciparum* and *P. vivax* are the predominant species, with a slightly higher prevalence of the latter especially on the southern islands [8]. Since the early 1990s, transmission rates have decreased as a result of malaria control measures and general improvement in health of the community [9,10]. On the southernmost island of Aneityum, a comprehensive elimination program was initiated in 1991 and elimination was achieved with a high degree of commitment from the local community in 1999 [11]. The Aneityum Project served as a proof of principle for the intensification of the malaria control program with the ultimate goal of elimination [9,10].

Previous population genetics studies of *P. falciparum* and the malaria vector *Anopheles farauti s.s.* in Vanuatu showed that populations were largely isolated on individual islands, with little gene flow among islands [12,13]. These findings implied that malaria control measures might be carried out on an island-by-island basis, which is the strategy currently used in the Pacific [10].

Merozoite surface protein 1 (MSP1) and circumsporozoite protein (CSP) are major surface antigens in *P. falciparum* and *P vivax*. These antigens are highly polymorphic, making them useful markers for assessment of parasite genetic diversity [7]. Earlier we examined *msp1* and *csp* polymorphisms in parasites from Vanuatu in the context of vaccine development for *P. falciparum* [14] and persisting humoral immunity after elimination on Aneityum Island for *P. vivax* [5]. In this study, using *msp1* and *csp* data previously generated for other aspects of malaria control, we compared the patterns of gene flow and population genetic structures in *P. falciparum* and *P. vivax* from seven sites on five islands in Vanuatu, and discussed the implications of our results in relation to the current malaria elimination strategy.

Materials and Methods

Ethics Statement

This study was approved by the Ministry of Health in Vanuatu and the Ethical Research Committee of Karolinska Institutet in Sweden. Due to the lack of a standardized writing system for the local "kastom" languages in Vanuatu, verbal informed consent was obtained from all adult participants and legal guardians in the case of minors. All pertinent information about the study, including the purpose, procedures, risks, benefits, and alternatives to participation, was provided to potential participants in both Bislama (lingua franca in Vanuatu; understood by most school-aged children and adults) by AK and the "kastom" language (understood by all participants) by local interpreters. The consent procedure was witnessed by a third party (e.g. teacher, village chief, nurse from local dispensary), who also recorded the name of each

participant as he/she enrolled in the study. The Ministry of Health in Vanuatu and the Ethical Research Committee of Karolinska Institutet in Sweden approved the use of this consent procedure.

Sample collections

P. falciparum and *P. vivax* isolates were collected during malariometric surveys conducted at seven sites on five islands (Gaua, Santo, Pentecost, Malakula, and Tanna) from five provinces in Vanuatu between 1996 and 2002 [5,14] (Fig. 1). Finger-pricked blood samples were collected on Whatman 31ET Chr filter paper (Whatman, Maidstone, UK) and stored desiccated [5].

DNA extractions, PCR amplifications, and genotyping/sequences

A subset of microscopy-positive samples from each site was randomly selected for this study. Genomic DNA was extracted from blood spotted on filter paper using the QIAamp DNA Blood Mini Kit (QIAgen, Germantown, MD) according to the manufacturer's instructions. PCR amplifications, and genotyping and/or sequencing of the merozoite surface protein 1 (*msp1*) and the circumsporozoite protein (*csp*) genes in *P. falciparum* [14,15] and *P. vivax* [5,16] were described previously. For each locus, samples with multiple alleles or genotypes were excluded for molecular analyses. The sequences described in this study have been deposited in the GenBank database (accession numbers AB116596-AB116607, AB539022-AB539045, and AB539540-AB539553).

Molecular analyses

For each locus, unbiased haplotype diversity (H) for each site was calculated using the equation $H = n(1 - \Sigma X_i^2)/(n-1)$, where n is the number of haplotypes and X_i is the frequency of the i-th haplotype [17].

Gene flow among populations was examined at two different levels. First, gene flow was examined among the seven sites. Second, populations from the same islands were pooled and gene flow was examined among the five islands.

For each locus, pairwise F_{ST} genetic distances among sites were estimated using the program Arlequin 3.5 [18]. Genetic distances for *P. falciparum* and *P. vivax* were based on the frequencies of shared haplotypes defined by sequence polymorphisms. The statistical significance of F_{ST} distances was evaluated by randomly permuting haplotypes between sites approximately 10,000 times to generate a null distribution against which the observed value was compared. Gene flow between sites was inferred when the pairwise F_{ST} genetic distance was not statistically significant ($p > 0.05$).

Genetic variation partitioned within populations, between populations within islands, and among islands was estimated by analysis of molecular variance (AMOVA) using the program Arlequin 3.5 [18]. The statistical significance of the observed values was evaluated by randomly permuting sequences among sites approximately 1,000 times to generate a null distribution against which the observed values were compared.

Results

P. falciparum and *P. vivax* infections

Overall, PCR amplifications of *msp1* and *csp* revealed more *P. falciparum* infections among the seven sites in Vanuatu (Table 1). *P. falciparum* was the predominant species in our samples from Pentecost and Malakula (Table 1). Different PCR efficacies between the *msp1* and the *csp* amplifications were likely the cause for the slightly different numbers of infections detected in

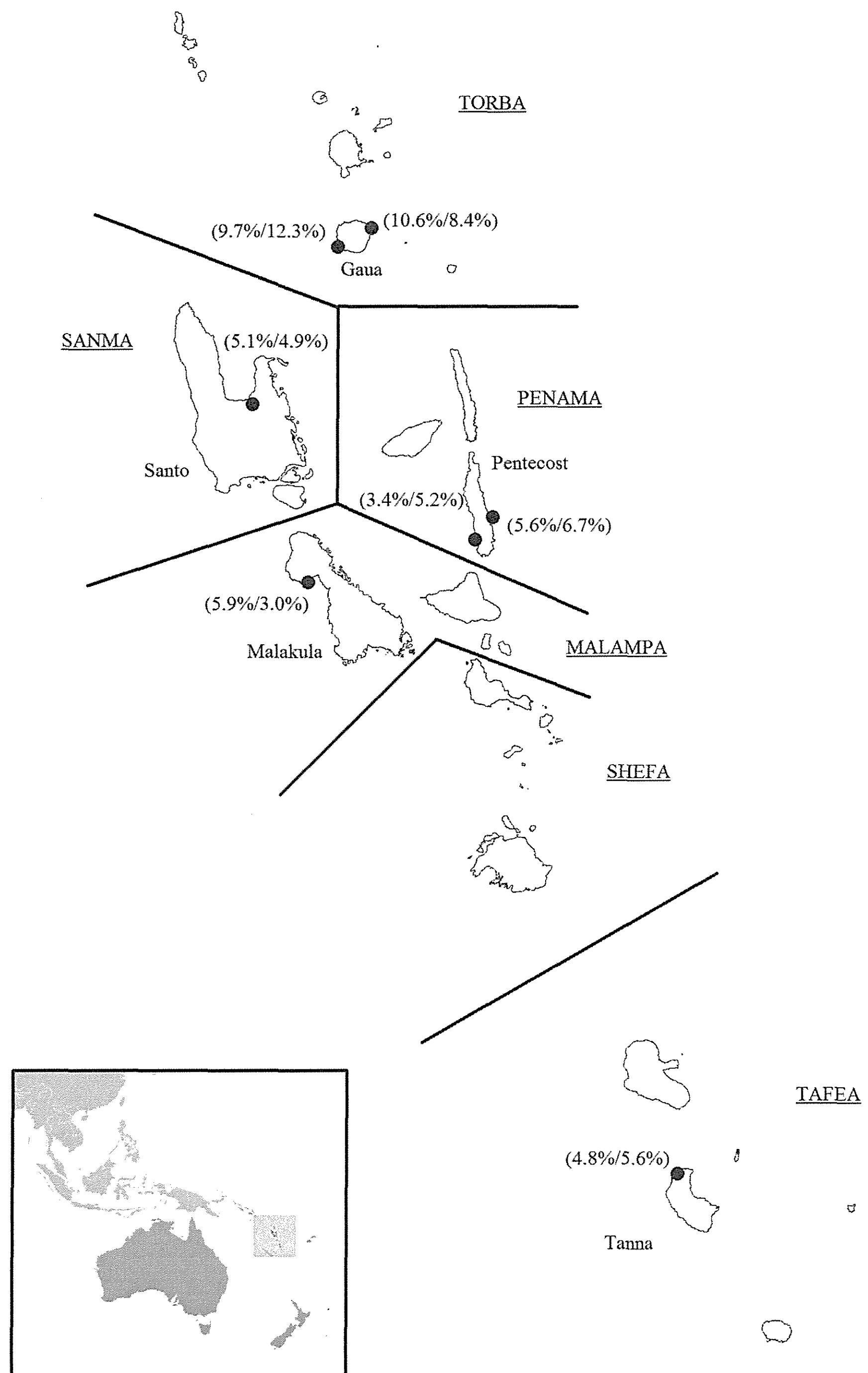


Fig 1. Map of Vanuatu showing the seven collection sites (black circles) on five islands. The names of the six provinces in Vanuatu are capitalized and underlined, and approximate provincial boundaries are indicated by solid lines. Species-specific parasite rates (*P. falciparum*/*P. vivax*) for each site were determined by microscopy. Maps were provided by the Library at the CIA (regional) and DIVA-GIS (Vanuatu).

doi:10.1371/journal.pone.0119475.g001

each site (Table 1). Multiple-genotype infections were more common in *P. vivax* than *P. falciparum* for both *msp1* (13.8% vs. 4.1%) and *csp* (36.2% vs. 3.6%) (Table 1).

Table 1. Numbers of merozoite surface protein 1 (*msp1*) and circumsporozoite protein (*csp*) sequences from seven sites in Vanuatu.

Site	*msp1* *P. falciparum*	*msp1* *P. vivax*	*csp* *P. falciparum*	*csp* *P. vivax*
East Gaua	16 (3)	23 (1)	19 (0)	14 (8)
West Gaua	14 (0)	10 (3)	10 (0)	6 (7)
Santo	24 (2)	27 (4)	21 (0)	23 (6)
East Pentecost	25 (1)	12 (2)	26 (0)	10 (9)
West Pentecost	16 (0)	3 (2)	14 (0)	2 (4)
Malakula	62 (1)	14 (4)	61 (6)	12 (7)
Tanna	8 (0)	11 (0)	8 (0)	7 (1)
Total	165 (7)	100 (16)	159 (6)	74 (42)

The numbers of infection with multiple genotypes are given in parentheses.

doi:10.1371/journal.pone.0119475.t001

P. falciparum and *P. vivax* genetic diversities

Genotyping and sequencing of *msp1* and *csp* revealed that *P. vivax* was more genetically diverse than *P. falciparum* in our samples from Vanuatu. In *P. falciparum*, six *msp1* and five *csp* haplotypes were observed, whereas in *P. vivax* 14 *msp1* and 20 *csp* haplotypes were observed (Tables A-D in S1 File). All *P. falciparum* isolates from Tanna (n = 8) were genetically identical for both *msp1* and *csp* (Tables A and B in S1 File). In *P. falciparum*, *msp1* diversities ranged from 0 in Tanna to 0.692 in West Gaua, while *csp* diversities ranged from 0 in East Pentecost and Tanna to 0.733 in West Gaua (Table 2). Very few *P. vivax* isolates were successfully genotyped in West Pentecost (Table 1), resulting in the extreme difference in diversity estimates between the two loci (0 for *msp1* vs. 1 for *csp*; Table 2). Excluding this site, *msp1* diversities ranged from 0.697 in East Pentecost to 0.889 in West Gaua, while *csp* diversities ranged from 0.822 in East Pentecost to 0.952 in Tanna (Table 2). Haplotype diversities were significantly higher in *P. vivax* than *P. falciparum* for both *msp1* (*t*-test; $p = 0.0135$) and *csp* ($p = 0.004$) when *P. vivax* from West Pentecost was excluded for comparison.

Patterns of gene flow

Seven-site analyses. Analyses of F_{ST} genetic distances showed that gene flow among *P. falciparum* populations was restricted. Between populations, gene flow in *msp1* was limited to those from the same islands (Gaua and Pentecost; Table 3), while gene flow in *csp* was observed between the two populations on Gaua, between West Gaua and Malakula, and between Tanna and the two populations on Pentecost (Table 3).

Table 2. Haplotype diversities of *msp1* and *csp* in *P. falciparum* and *P. vivax* from seven sites in Vanuatu.

Site	*msp1* *P. falciparum*	*msp1* *P. vivax*	*csp* *P. falciparum*	*csp* *P. vivax*
East Gaua	0.6583	0.8854	0.5906	0.8791
West Gaua	0.6923	0.8889	0.7333	0.8667
Santo	0.6703	0.8234	0.5286	0.9012
East Pentecost	0.5133	0.6970	0.0000	0.8222
West Pentecost	0.4583	0.0000	0.3626	1.0000
Malakula	0.5600	0.8791	0.7098	0.8788
Tanna	0.0000	0.7091	0.0000	0.9524

doi:10.1371/journal.pone.0119475.t002

Table 3. Pairwise F_{ST} genetic distances based on *msp1* (lower triangle) and *csp* (upper triangle) haplotype frequencies in *P. falciparum* from seven sites in Vanuatu.

Site	East Gaua	West Gaua	Santo	East Pentecost	West Pentecost	Malakula	Tanna
East Gaua		−0.001*	0.100	0.614	0.346	0.188	0.463
West Gaua	−0.065*		0.160	0.647	0.301	0.055*	0.440
Santo	0.308	0.288		0.759	0.509	0.275	0.646
East Pentecost	0.193	0.217	0.386		0.240	0.327	0.000*
West Pentecost	0.338	0.340	0.391	0.068*		0.133	0.088*
Malakula	0.106	0.142	0.324	0.067	0.251		0.251
Tanna	0.591	0.585	0.209	0.645	0.708	0.512	

Gene flow as defined by non-statistically significant ($p > 0.05$) F_{ST} distance is indicated by an asterisk (*).

doi:10.1371/journal.pone.0119475.t003

In contrast, a greater degree gene flow among *P. vivax* populations was observed. Examination of the *msp1* F_{ST} distances revealed gene flow among all *P. vivax* populations in northern and central Vanuatu except those between East Gaua and West Pentecost (Table 4). The population in Tanna remained genetically distinct from all other populations, however (Table 4). For *csp*, gene flow was observed among all populations on Santo, Pentecost, Malakula, and Tanna. The two populations on Gaua were genetically distinct from those in Santo and East Pentecost. Also, East Gaua was genetically distinct from Malakula, while West Gaua was distinct from Tanna (Table 4).

Five-island analyses. Analyses of F_{ST} distances among islands revealed patterns of gene flow consistent with those from the seven-site analyses. For *P. falciparum*, gene flow among populations on different islands was very minimal. *P. falciparum* populations in central Vanuatu (Santo, Pentecost, and Malakula) were significantly differentiated from one another, despite relatively small distances separating these islands. Gene flow between Pentecost and Tanna was observed as a result of the significant sharing of the 42NE haplotype in *csp* (Fig. 2; Table B in S1 File). For *P. vivax*, gene flow among populations on different islands was more widespread. Gene flow among islands in central Vanuatu (Santo, Malakula, and Pentecost) was evident in both *msp1* and *csp* (Fig. 2). However, gene flow between the peripheral islands of Gaua and Tanna and the central islands was more limited. For Gaua, gene flow with Santo and Malakula was observed for *msp1* only, while for Tanna, gene flow with all other islands was observed for *csp* only (Fig. 2).

Table 4. Pairwise F_{ST} genetic distances based on *msp1* (lower triangle) and *csp* (upper triangle) haplotype frequencies in *P. vivax* from seven sites in Vanuatu.

Site	East Gaua	West Gaua	Santo	East Pentecost	West Pentecost	Malakula	Tanna
East Gaua		0.034*	0.075	0.123	0.056*	0.094	0.017*
West Gaua	−0.005*		0.073	0.129	0.094*	0.089*	0.089
Santo	0.014*	0.011*		−0.004*	0.008*	0.001*	0.028*
East Pentecost	0.056*	0.041*	−0.029*		0.132*	0.045*	0.063*
West Pentecost	0.203	0.164*	0.210*	0.193*		−0.043*	-0.041*
Malakula	0.003*	−0.023*	−0.034*	−0.028*	0.166*		0.065*
Tanna	0.164	0.114	0.205	0.264	0.438	0.170	

Gene flow as defined by non-statistically significant ($p > 0.05$) F_{ST} distance is indicated by an asterisk (*).

doi:10.1371/journal.pone.0119475.t004

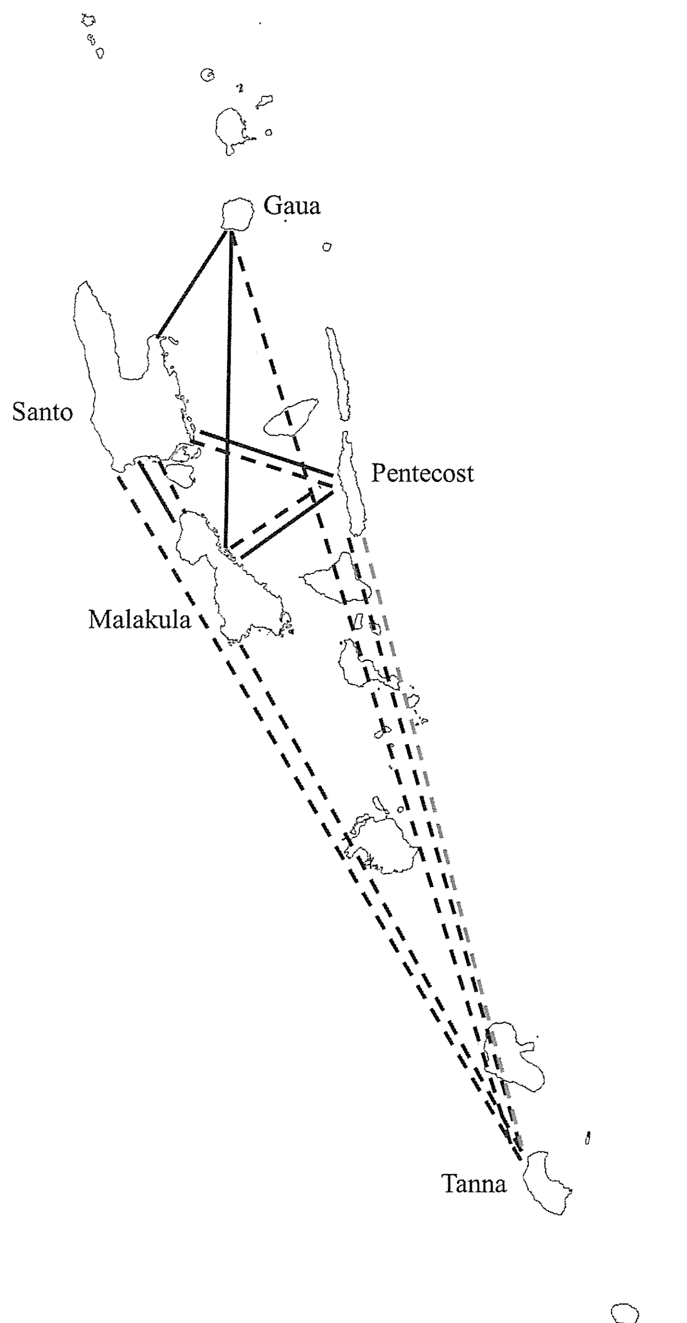


Fig 2. Gene flow among *P. falciparum* (gray) and *P. vivax* (black) populations from five islands in Vanuatu. Solid lines represent inferred gene flow based on merozoite surface protein 1 (*msp1*) F_{ST} genetic distances, while dotted lines represent gene flow based on circumsporozoite protein (*csp*) distances. No gene flow was observed in *P. falciparum msp1*. The map of Vanuatu was provided by DIVA-GIS.

doi:10.1371/journal.pone.0119475.g002

Partitioning of genetic variation

Different population genetic structures for *P. falciparum* and *P. vivax* were revealed by AMOVA (Table 5). For *P. falciparum*, while most of the genetic variation (66.7–70.7%) was found within sites, variation among islands was substantial (28.8–31.2%) and statistically significant ($p = 0.014$ for *msp1* and $p = 0.028$ for *csp*). This is consistent with the F_{ST} genetic

Table 5. Percentages of genetic variance partitioned at different population levels using analysis of molecular variance (AMOVA).

	P. falciparum	***P. falciparum***	***P. vivax***	***P.vivax***
Source of variation	*msp1*, %	*csp*, %	*msp1*, %	*csp*, %
Among islands	28.81*	31.23*	3.99	-0.53
Within island, between sites	0.53	2.06	2.93	5.77
Within sites	70.66	66.71	93.07	94.77

* $p < 0.05$

doi:10.1371/journal.pone.0119475.t005

distance analyses described above that showed significant genetic differentiation among most islands (Fig. 2). In contrast, almost all genetic variation (93.1–94.8%) in *P. vivax* was found within sites, and the lack of significant variation among islands ($p = 0.221$ for *msp1* and $p = 0.510$ for *csp*) was in agreement with gene flow among islands as revealed in the F_{ST} genetic distance analyses (Fig. 2).

Discussion

In Vanuatu, *P. falciparum* and *P. vivax* are the major malaria species, with a slight predominance of the latter [8]. In our samples we observed more *P. falciparum* than *P. vivax* infections, especially on Pentecost and Malakula (Table 1). Such difference might reflect the seasonal fluctuations in species prevalence in Vanuatu. Malaria transmission in Vanuatu is perennial. While incidence of *P. vivax* shows little seasonal fluctuation, incidence of *P. falciparum* peaks during the rainy season, from November to April [8].

Despite a slightly higher prevalence in our samples, *P. falciparum* showed consistently less genetic diversity than *P. vivax* in both *msp1* and *csp* across all sites except in West Pentecost, where few *P. vivax* isolates were found (Tables 1 and 2). Structural difference in orthologous genes between these two species may partially account for the difference in genetic diversity observed. For example, in *P. falciparum* sequence variation in *msp1* is dimorphic (either K1 or MAD 20 allelic type) and much of the variation is limited to the presence (or absence) and length of unique nine base-pair repeats in block 2 [19]. In contrast, *msp1* in *P. vivax* contains multiple variable blocks with extensive variation in repeats and nucleotide substitutions, and numerous potential recombination sites within and between variable blocks [20]. Direct comparison of genetic diversity in these orthologous loci between species may not be straightforward, nonetheless our result of lower genetic diversity in *P. falciparum* than in sympatric *P. vivax* was consistent with previous studies using neutral microsatellites [21,22] and other surface antigens such as apical membrane antigen 1 (*ama1*) [23,24].

Compared to *P. falciparum*, multiple-genotype infections were more common in *P. vivax* (Table 1). High frequencies of multiple-genotype infections facilitate meiotic recombination in the *Anopheles* mosquito vectors, leading to generation of novel genotypes [25] and greater genetic diversity in *P. vivax* (Table 2). In our *P. vivax* samples, the higher frequency of multiple-genotype infections in *csp* vs. *msp1* (36.2% vs. 13.8%; Table 1) was consistent with previous results from Thailand [26] and India [27].

Both MSP1 and CSP are major surface antigens, and high levels of polymorphisms in these loci are known to be a result of selection by host immunity [7,14]. However, it remains unclear whether the selective pressure on, and by extension genetic diversity in the orthologous loci of *P. falciparum* and *P. vivax* are directly comparable [7]. Differential *msp1* and *csp* genetic diversity in our samples might reflect differential selection by host immunity, i.e. stronger immune selection on the *P. falciparum* orthologs reduced genetic diversity observed in our samples.

Moreover, specific host immune response to MSP1 and CSP may differ between *P. falciparum* and *P. vivax*, resulting in different patterns of selection seen among the orthologs [28,29]. It has been shown that in *P. vivax*, rapid expansion and contraction of repeats in *csp* by slipped-strand mispairing was driven by immune selection [29], consistent with our observation of related and near-identical *csp* haploytpes (e.g. VC06/13/16/19; Table D in S1 File) and high frequencies of *csp* multiple-genotype infections (Table 1).

At the global level different evolutionary histories of *P. falciparum* and *P. vivax* likely contributed to the high level of genetic diversity seen in the latter [30], however in Vanuatu the role of population history in shaping parasite genetic diversity is not well understood. *P. vivax* is believed to have accompanied *Homo sapiens* when the latter first settled the Pacific > 40,000 years before present (ybp), compared to the relatively recent arrival of *P. falciparum* within the last 10,000 years [31–33]. However, northern Vanuatu was first settled only 3200 ybp by Lapita migrants from the Solomon Islands [34], suggesting that both *P. falciparum* and *P. vivax* were introduced to Vanuatu at the same time [8,31,32]. Despite similar time depth within Vanuatu, the founder effect associated with the initial colonization might have been different between the two parasite species. Previous studies on *P. falciparum* and *P. vivax* population genetics showed a decrease in *P. falciparum* microsatellite genetic diversity in Temotu Province of the Solomon Islands when compared to Papua New Guinea, but no decrease in *P. vivax* [21,35], suggesting that the effective (reproductive) population sizes of the founding populations in the Solomon Islands might have been different between these two parasite species. To the southeast of the Solomon Islands, the initial introduction of malaria parasites to Vanuatu represents yet another founding event. Our observation of lower genetic diversity in *P. falciparum* from Vanuatu is consistent with the results from Temotu Province in the Solomon Islands [21], which was also first settled by Lapita migrants about 3200 ybp [36], further supporting the idea that genetic drift (founder effect) might have played a greater role in shaping the genetic diversity of *P. falciparum* than that of *P. vivax* in Vanuatu.

In Vanuatu, inter-island gene flow likely contributed to the higher genetic diversity in *P. vivax* populations in two ways. First, gene flow mitigates the loss of haplotypes due to genetic drift in isolated island populations. In *P. vivax*, the most abundant *msp1* and *csp* haplotypes were shared among all five sampled islands, whereas in *P. falciparum* no *msp1* and *csp* haplotypes were shared by more than three and four islands, respectively (Tables A and B in S1 File). Second, maintenance of distinct haplotypes in a population allows for generation of novel haplotypes by recombination. For example, in *P. vivax msp1* haplotypes VM03 and VM08 might have arisen from a recombination event between haplotypes VM01 and VM06 on Malakula, where all four lineages were found (Table C in S1 File). Recombination within the poly-Q sequence in block 6 of *msp1* might have further enhanced the polymorphic nature of the gene in *P. vivax* (Table C in S1 File). In contrast, limited recombination events [19] as a result of isolation shown here and previously [12] might have contributed to the relatively lower level of genetic diversity observed in *P. falciparum*.

Gene flow in *P. vivax* might be facilitated by its ability to form dormant hypnozoites in the host liver and the rapid development and emergence of gametocytes. Anti-hypnozoite treatment with primaquine is not usually administered to local *P. vivax* cases in Vanuatu [5]. Furthermore, unlike those with blood-stage parasites, *P. vivax* hypnozoite-carriers are asymptomatic and might therefore be less averse to long-distance travel (e.g. between islands). Once activated, latent hypnozoites develop into merozoites, which invade red blood cells to start the erythrocytic cycle of infection. In contrast to *P. falciparum*, *P. vivax* gametocytes are known to develop early, often before symptoms appear and treatments are sought, making *P. vivax* transmission efficient and persistent [37,38]. The period of extrinsic development of *P. vivax* is known to be shorter than that of *P. falciparum* [39], which may further facilitate *P. vivax*

transmission. In Vanuatu, *An. farauti s.s.* is the sole malaria vector [13]. It is unknown whether the efficiency with which this vector transmits parasites is different between *P. falciparum* and *P. vivax*, and how this difference, if it exists, might affect gene flow and genetic diversity.

Even though *P. vivax* showed a greater degree of gene flow, the extent of parasite movement appears to be distance dependent. Gene flow among *P. vivax* populations from the central islands of Santo, Malakula, and Pentecost was observed for both *msp1* and *csp*, while populations from the peripheral islands of Gaua and Tanna were more isolated, showing gene flow with these central island populations in only one locus. Parasite movement among Santo, Malakula, Pentecost, and to a less extent Gaua, is consistent with the existence of traditional exchange networks in northern and central Vanuatu, where both cultural (e.g. shell, pottery, mats) and biological (e.g. kava, yams, pigs) items are transported and traded across many islands [40]. It is reasonable to hypothesize that *P. vivax* is also transported and exchanged among these islands, albeit unintentionally. Tanna is not known to be a part of the aforementioned traditional exchange networks, instead parasite movement and gene flow between Tanna and these other islands might reflect the recent convenience of interisland air travel [9].

As samples used in this study were collected over a span of six years (1996 to 2002), potential temporal variation in parasite populations should be considered in the interpretation of parasite genetic diversity and gene flow. We evaluated the temporal "stability" of parasite populations from four sites (Santo, Malakula, East and West Pentecost) in which there were samples from at least two years. Analyses of *msp1* and *csp* F_{ST} genetic distances revealed no year-to-year differentiation among *P. vivax* populations from the same site, indicating that *P. vivax* populations remained relatively stable over the sampled period. For *P. falciparum*, genetic differentiation was observed among temporal populations from Malakula (both *msp1* and *csp*) and East Pentecost (*msp1* only). Given that *P. falciparum* incidence in Vanuatu shows strong seasonality [8], drastic year-to-year changes in the genetic makeup of *P. falciparum* populations due to genetic drift during the dry season are not unexpected. More comprehensive sampling of contemporaneous parasite populations from different islands will allow for a more refined description of gene flow in both *P. falciparum* and *P. vivax*.

Distinct parasite population structures and patterns of gene flow between *P. falciparum* and *P. vivax* have important implications on the current malaria initiatives in Vanuatu. Our previous analyses of *P. falciparum* and *An. farauti s.s.* genetic diversities showed that these two species were largely localized to individual islands [12,13]. However for *P. vivax*, we demonstrated that parasite movement among islands and across provincial boundaries is common, suggesting that the current island-by-island elimination strategy might need to be complemented with more integrated control and coordination among islands and provinces [10]. Moreover, the risk of resurgence or reintroduction of parasites from other islands after elimination should not be underestimated, as shown by our own experience on Aneityum Island, where *P. vivax* from Tanna was responsible for the outbreak six years after initial elimination [5].

Conclusions

In Vanuatu, *P. falciparum* and *P. vivax* were both present but showed different levels of genetic diversity and different patterns of gene flow and population structures. The high level of diversity in *P. vivax* populations was maintained by greater degree of gene flow among islands, which also resulted in greater genetic similarity among populations on different islands. Our data suggested that the current malaria control strategy might need to be bolstered with centrally integrated components and coordination among islands and provinces to ensure elimination and sustainable malaria freedom.

Supporting Information

S1 File. Distributions of merozoite surface surface protein 1 (msp1) and circumsporozoite protein (csp) haplotypes in *Plasmodium falciparum* and *Plasmodium vivax* from seven sites in Vanuatu.
(XLSX)

Acknowledgments

The authors would like to express their sincere gratitude to the study participants and the local survey assistants on various islands, and George Taleo, Morris Kalkoa, James Yaviong, Hope Leodoro, Sam Yamar, and Peter Kalcei from the Ministry of Health in Vanuatu.

Author Contributions

Conceived and designed the experiments: JKL KT AK. Performed the experiments: NS SIT KT. Analyzed the data: CWC NS SIT ZMI JKL KT. Contributed reagents/materials/analysis tools: NS SIT JKL KT AK. Wrote the paper: CWC NS SIT ZMI JKL AK.

References

1. Feachem RG, Phillips AA, Hwang J, Cotter C, Wielgosz B, Greenwood BM, et al. Shrinking the malaria map: progress and prospects. Lancet 2010; 376:1566–1578. doi: 10.1016/S0140-6736(10)61270-6 PMID: 21035842
2. Alonso PL, Tanner M. Public health challenges and prospects for malaria control and elimination. Nat Med 2013; 19:150–155. doi: 10.1038/nm.3077 PMID: 23389615
3. Price RN, Tjitra E, Guerra CA, Yeung S, White NJ, Anstey NM. Vivax malaria: neglected and not benign. Am J Trop Med Hyg 2007; 77(6 Suppl):79–87. PMID: 18165478
4. Cohen JM, Smith DL, Cotter C, Ward A, Yamey G, Sabot OJ, et al. Malaria resurgence: a systematic review and assessment of its causes. Malar J 2012; 11:122. doi: 10.1186/1475-2875-11-122 PMID: 22531245
5. Kaneko A, Chaves LF, Taleo G, Kalkoa M, Isozumi R, Wickremasinghe R, et al. Characteristic age distribution of *Plasmodium vivax* infections after malaria elimination on Aneityum Island. Infect Immun 2014; 82:243–252. doi: 10.1128/IAI.00931-13 PMID: 24166950
6. Murhandarwati EE, Fuad A, Nugraheni MD, Suyanto S, Wijayanti MA, Widartono BS, et al. Early malaria resurgence in pre-elimination areas in Kokap Subdistrict, Kulon Progo, Indonesia. Malar J 2014; 13:130. doi: 10.1186/1475-2875-13-130 PMID: 24684702
7. Arnott A, Barry AE, Reeder JC. Understanding the population genetics of *Plasmodium vivax* is essential for malaria control and elimination. Malar J 2012; 11:14. doi: 10.1186/1475-2875-11-14 PMID: 22233585
8. Kaneko A, Taleo G, Kalkoa M, Yaviong J, Reeve PA, Ganczakowski M, et al. Malaria epidemiology, glucose 6-phosphate dehydrogenase deficiency and human settlement in the Vanuatu Archipelago. Acta Trop 1998; 70:285–302. PMID: 9777715
9. Pacific Malaria Initiative Survey Group (PMISG) on behalf of the Ministries of Health of Vanuatu and Solomon Islands. Malaria on isolated Melanesian islands prior to the initiation of malaria elimination activities. Malar J 2010; 9:218. doi: 10.1186/1475-2875-9-218 PMID: 20659316
10. Asia Pacific Malaria Elimination Network. Country briefing: eliminating malaria in Vanuatu. 2013. Available: http://apmen.org/storage/country-briefings/Vanuatu.pdf.
11. Kaneko A, Taleo G, Kalkoa M, Yamar S, Kobayakawa T, Björkman A. Malaria eradication on islands. Lancet 2000; 356:160–164. PMID: 10963263
12. Lum JK, Kaneko A, Taleo G, Amos M, Reiff DM. Genetic diversity and gene flow of humans, *Plasmodium falciparum*, and *Anopheles farauti s.s.* of Vanuatu: inferred malaria dispersal and implications for malaria control. Acta Trop 2007; 103:102–107. PMID: 17662681
13. Reiff DM, Kaneko A, Taleo G, Amos M, Lum JK. Population structure and gene flow of *Anopheles farauti s.s.* (Diptera: Culicidae) among ten sites on five islands of Vanuatu: implications for malaria control. J Med Entomol 2007; 44:601–607. PMID: 17695014

14. Tanabe K, Sakihama N, Kaneko A. Stable SNPs in malaria antigen genes in isolated populations. Science 2004; 303:493. PMID: 14739451

15. Sakihama N, Mitamura T, Kaneko A, Horii T, Tanabe K. Long PCR amplification of *Plasmodium falciparum* DNA extracted from filter paper blots. Exp Parasitol 2001; 97:50–54. PMID: 11207114

16. Tanabe K, Escalante A, Sakihama N, Honda M, Arisue N, Horii T, et al. Recent independent evolution of *msp1* polymorphism in *Plasmodium vivax* and related simian malaria parasites. Mol Biochem Parasitol 2007; 156:74–79. PMID: 17706800

17. Nei M. Molecular evolutionary genetics. New York: Columbia University Press; 1987.

18. Excoffier L, Lischer HEL. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour 2010; 10:564–567. doi: 10.1111/j.1755-0998.2010.02847.x PMID: 21565059

19. Sakihama N, Kaneko A, Hattori T, Tanabe K. Limited recombination events in merozoite surface protein-1 alleles of *Plasmodium falciparum* on islands. Gene 2001; 279:41–48. PMID: 11722844

20. Putaporntip C, Jongwutiwes S, Sakihama N, Ferreira MU, Kho WG, Kaneko A, et al. Mosaic organization and heterogeneity in frequency of allelic recombination of the *Plasmodium vivax* merozoite surface protein-1 locus. Proc Natl Acad Sci USA 2002; 99:16348–16353. PMID: 12466500

21. Gray KA, Dowd S, Bain L, Bobogare A, Wini L, Shanks GD, et al. Population genetics of *Plasmodium falciparum* and *Plasmodium vivax* and asymptomatic malaria in Temotu Province, Solomon Islands. Malar J 2013; 12:429. doi: 10.1186/1475-2875-12-429 PMID: 24261646

22. Orjuela-Sánchez P, Sá JM, Brandi MC, Rodrigues PT, Bastos MS, Amaratunga C, et al. Higher microsatellite diversity in *Plasmodium vivax* than in sympatric *Plasmodium falciparum* populations in Pursat, Western Cambodia. Exp Parasitol 2013; 134:318–326. doi: 10.1016/j.exppara.2013.03.029 PMID: 23562882

23. Ord RL, Tami A, Sutherland CJ. *ama1* genes of sympatric *Plasmodium vivax* and *P. falciparum* from Venezuela differ significantly in genetic diversity and recombination frequency. PLoS One 2008; 3: e3366. doi: 10.1371/journal.pone.0003366 PMID: 18846221

24. Arnott A, Wapling J, Mueller I, Ramsland PA, Siba PM, Reeder JC, et al. Distinct patterns of diversity, population structure and evolution in the AMA1 genes of sympatric Plasmodium falciparum and Plasmodium vivax populations of Papua New Guinea from an area of similarly high transmission. Malar J 2014; 13:233. doi: 10.1186/1475-2875-13-233 PMID: 24930015

25. Kerr PJ, Ranford-Cartwright LC, Walliker D. Proof of intragenic recombination in *Plasmodium falciparum*. Mol Biochem Parasitol 1994; 66:241–248. PMID: 7808474

26. Imwong M, Pukrittayakamee S, Grüner AC, Rénia L, Letourneur F, Looareesuwan S, et al. Practical PCR genotyping protocols for Plasmodium vivax using Pvcs and Pvmsp1. Malar J 2005; 4:20. PMID: 15854233

27. Kim JR, Imwong M, Nandy A, Chotivanich K, Nontprasert A, Tonomsing N, et al. Genetic diversity of Plasmodium vivax in Kolkata, India. Malar J 2006; 5:71. PMID: 16907979

28. Pacheco MA, Poe AC, Collins WE, Lal AA, Tanabe K, Kariuki SK, et al. A comparative study of the genetic diversity of the 42kDa fragment of the merozoite surface protein 1 in *Plasmodium falciparum* and *P. vivax*. Infect Genet Evol 2007; 7:180–187. PMID: 17010678

29. Parobek CM, Bailey JA, Hathaway NJ, Socheat D, Rogers WO, Juliano JJ. Differing patterns of selection and geospatial genetic diversity within two leading *Plasmodium vivax* candidate vaccine antigens. PLoS Negl Trop Dis 2014; 8:e2796. doi: 10.1371/journal.pntd.0002796 PMID: 24743266

30. Neafsey DE, Galinsky K, Jiang RH, Young L, Sykes SM, Saif S, et al. The malaria parasite *Plasmodium vivax* exhibits greater genetic diversity than *Plasmodium falciparum*. Nat Genet 2012; 44:1046–1050. doi: 10.1038/ng.2373 PMID: 22863733

31. Groube LM. Contradictions and malaria in Melanesian and Australian prehistory. In: Spriggs M, Yen D, Ambrose W, Jones R, Thorne A, Andrews A, editors. A Community of Culture: The People and Prehistory of the Pacific. Canberra: Department of Prehistory, Australian National University; 1993. pp. 164–186.

32. Clark JT, Kelly KM. Human genetics, paleoenvironments, and malaria: relationships and implications for the settlement of Oceania. Am Anthropol 1993; 95:612–630.

33. Buckley HR. 'The predators within': investigating the relationship between malaria and health in the prehistoric Pacific Islands. In: Oxenham M, Tales N, editors. Bioarchaeology of Southeast Asia. Cambridge: Cambridge University Press; 2005. pp. 309–332.

34. Hedrick J. Lapita style pottery from Malo Island. J Polyn Soc 1971; 80:5–19.

35. Anderson TJ, Haubold B, Williams JT, Estrada-Franco JG, Richardson L, Mollinedo R, et al. Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. Mol Biol Evol 2000; 17:1467–1482. PMID: 11018154

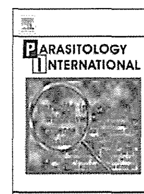
36. Sheppard PJ, Walter R. A revised model of Solomon Islands culture history. J Polyn Soc 2006; 115:47–76.
37. Bousema T, Drakeley C. Epidemiology and infectivity of *Plasmodium falciparum* and *Plasmodium vivax* gametocytes in relation to malaria control and elimination. Clin Microbiol Rev 2011; 24:377–410. doi: 10.1128/CMR.00051-10 PMID: 21482730
38. Galinski MR, Meyer EV, Barnwell JW. *Plasmodium vivax*: modern strategies to study a persistent parasite's life cycle. Adv Parasitol 2013; 81:1–26. doi: 10.1016/B978-0-12-407826-0.00001-1 PMID: 23384620
39. Macdonald G. The epidemiology and control of malaria. London: Oxford University Press; 1957.
40. Huffman K. Trading, cultural exchange and copyright: important aspects of Vanuatu arts. In: Bonnemaison J, Huffman K, Kaufmann C, Tryon D, editors. Arts of Vanuatu. Bathurst: Crawford House Press; 1996. pp. 182–194.

Parasitology International 64 (2015) 304–308

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint



Improved detection of malaria cases in island settings of Vanuatu and Kenya by PCR that targets the *Plasmodium* mitochondrial cytochrome c oxidase III (*cox3*) gene

Rie Isozumi [a], Mayumi Fukui [a], Akira Kaneko [a,b], Chim W. Chan [b], Fumihiko Kawamoto [c], Masatsugu Kimura [d,*]

[a] Department of Medical Zoology, Graduate School of Medicine, Osaka City University, 1-4-3, Asahi-machi, Abeno-ku, Osaka 545-8585, Japan
[b] Island Malaria Group, Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institutet, Nobels väg 16, SE 171 77 Stockholm, Sweden
[c] Division of International Health, Research Promotion Project, Oita University Faculty of Medicine, Yufu 879-5593, Japan
[d] Radioisotope Centre, Graduate School of Medicine, Osaka City University, 1-4-3, Asahi-machi, Abeno-ku, Osaka 545-8585, Japan

ARTICLE INFO

Available online 22 September 2014

Keywords:
Plasmodium
Human malaria
Mitochondrial DNA
cox3
Nested PCR
Malaria diagnosis

ABSTRACT

Detection of sub-microscopic parasitemia is crucial for all malaria elimination programs. PCR-based methods have proven to be sensitive, but two rounds of amplification (nested PCR) are often needed to detect the presence of *Plasmodium* DNA. To simplify the detection process, we designed a nested PCR method whereby only the primary PCR is required for the detection of the four major human *Plasmodium* species. Primers designed for the detection of the fifth species, *Plasmodium knowlesi*, were not included in this study due to the absence of appropriate field samples. Compared to the standard 18S rDNA PCR method, our cytochrome c oxidase III (*cox3*) method detected 10–50% more cases while maintaining high sensitivities (1.00) for all four *Plasmodium* species in our samples from Vanuatu (n = 77) and Kenya (n = 76). Improvement in detection efficiency was more substantial for samples with sub-microscopic parasitemia (54%) than those with observable parasitemia (10–16%). Our method will contribute to improved malaria surveillance in low endemicity settings.
© 2014 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Accurate and sensitive detection of parasitemia is an essential component of any malaria elimination program [1]. Microscopy has traditionally been the method of choice, but reliable results require microscopists with extensive training and experience, and lengthy examination time with samples that have very low parasitemias [2]. Detection of malaria antigens by rapid diagnostic tests (RDTs) is clinically useful, but not all RDTs are capable of identifying all human *Plasmodium* species, and false positive due to residual proteins after parasite clearance has been observed [3].

PCR-based methods that detect the presence of *Plasmodium* DNA have proved to be more sensitive than microscopy, especially in the case of co-infections with more than one *Plasmodium* species [2,4]. The original nested PCR method targeting the *Plasmodium* 18S rDNA [2] is the standard against which many subsequent PCR-based methods have been tested.

In our experience, there are two major drawbacks associated with PCR methods that target the 18S rDNA [2,5]. First, the amplification efficiency of the primary PCR reaction of the original 18S rDNA method is low, yielding no visible bands when products are examined by gel electrophoresis. To properly detect the presence of *Plasmodium* DNA, four independent nested PCRs are needed for all samples. This is both time- and resource-consuming for analyses of samples from low-transmission areas where most samples are expected to be negative. Second, the binding specificity of the *Plasmodium* 18S rDNA primers is low. In an attempt to increase the amplification efficiency of the primary PCR of the original 18S rDNA method, the number of cycles was increased from 25 to 35–40, but multiple products were generated. Our previous nested PCR method targeting a different region of the 18S rDNA also yielded multiple products after the primary PCR when it was performed for 35–40 cycles [6]. Together these results suggest that the human 18S rDNA present in samples was also amplified using the *Plasmodium* 18S rDNA primers because of sequence similarities between human and *Plasmodium* species. This can be problematic when human DNA is co-extracted with parasite DNA, as in the case of blood spot on filter papers. In light of these shortcomings, we sought to design a new PCR-based method with a different molecular target that allows for easier parasite detection and greater specificity.

The *Plasmodium* mitochondrial genome is an attractive target for PCR-based detection of parasitemia. First, the mitochondrial genome has higher copy numbers than the 18S rDNA. In each parasite, 20–150 copies of the 6-kilobase mitochondrial genome are found, whereas only 4–8 copies of the 18S rDNA are present [7–9]. A larger number of initial templates for PCR should allow for more efficient amplification, eliminate the need for the nested PCR to detect the presence of parasite DNA, and improve detection sensitivity. Second, the gene arrangement

* Corresponding author. Tel.: +81 6 66453950; fax: +81 6 66453952.
E-mail address: mkimura@med.osaka-cu.ac.jp (M. Kimura).

http://dx.doi.org/10.1016/j.parint.2014.09.006
1383-5769/© 2014 Elsevier Ireland Ltd. All rights reserved.

of the mitochondrial genome is highly conserved among species within the genus *Plasmodium*, and this gene arrangement is different from that of the human mitochondrial genome [10]. It is expected that, relative to the 18S rDNA, primers designed to target the *Plasmodium* mitochondrial genome should better minimize the simultaneous amplification of the human orthologous sequences.

A number of PCR-based detection methods targeting the mitochondrial genome have been proposed [4,11,12], however none has shown both simpler detection (i.e. single-round amplification) and greater detection sensitivity relative to the standard 18S rDNA method [2]. Here we describe a new PCR method that targets the cytochrome c oxidase III (*cox3*) region of the *Plasmodium* mitochondrial genome. Our method requires only a single round of PCR amplification for detection of *Plasmodium* DNA in blood samples collected from field, with greater detection sensitivity than the standard 18S rDNA method.

2. Materials and methods

2.1. Sample collections

Malariometric surveys were conducted on the mesoendemic island of Ambae and the hypoendemic island of Tanna in Vanuatu in 2002, and the hyperendemic islands of Lake Victoria in Kenya in 2012. *P. falciparum* and *P. malariae* are endemic in both Vanuatu and Kenya. In addition to these two species, *P. vivax* is endemic in Vanuatu while *P. ovale* is present in Kenya. Giemsa-stained blood smears were examined by experienced microscopists. Parasitemia was determined by counting the number of parasites against 100 (Vanuatu) or 200 (Kenya) leukocytes in the thick film. In Kenya, *P. falciparum* infections were also detected by Paracheck Pf® (Orchid, Goa, India) according to manufacturer's instructions.

Finger-pricked blood was collected on Whatman 31ET Chr filter paper (Whatman, Maidstone, UK) from residents on Ambae (n = 37) and Tanna (n = 40) in Vanuatu, and islands of Lake Victoria in Kenya (n = 76). Desiccated blood spots were stored in individual plastic bags at −20 °C until DNA extraction.

2.2. DNA extraction

Total DNA was extracted from three discs (6 mm in diameter) of blood spots using the QIAamp DNA Mini Kit (QIAgen, USA). DNA was eluted in 150 μl of the provided buffer.

2.3. Primer design

Whole mitochondrial sequences from 285 *P. vivax* (GenBank accession numbers AY598035-140, DQ396547-49, AY791517-692), 98 *P. falciparum* (M76611, DQ642845, and AY282924–3019), six *P. malariae* (AB354570, AB489192-4, and GQ355485-6), and one *P. ovale* (AB354571) samples were aligned using the software Genetyx V10 (Genetyx Corporation, Japan). The fifth species, *P. knowlesi*, has a natural host in the long-tailed macaques (*Macaca fascicularis*) and is endemic only in Southeast Asia [13]. Primers designed for this species were not explicitly tested with our field samples and are therefore not included in the current study.

A nested PCR using primers targeting the *Plasmodium cox3* genes was designed using the software Oligo Analyzer 1.5 (www.genelink.com). Primers for the primary PCR were genus specific, while those for the secondary PCR were species specific (Fig. 1). Genus specific primers were designed to avoid regions with high sequence similarity to human mtDNA, while each pair of the species specific primers was different from the other pairs by at least seven nucleotides at the 3′ ends.

A set of universal nested primers was designed to confirm the amplification of *Plasmodium cox3* by primary PCR in the case of ambiguous results (e.g. faint or multiple bands). They were also used to evaluate the ability of the primary PCR to detect all *Plasmodium* infections in a subset of samples (see below).

2.4. cox3 amplifications

The primary PCR was carried out in a 20 μl reaction containing 6 μl of template DNA (corresponding to 0.3 μl of blood), 0.2 μM of each primer (Fig. 1), and 10 μl of the PrimeSTAR Max DNA Polymerase Mix (Takara, Kyoto, Japan). Cycling conditions consisted of an initial activation at 96 °C for 1 min, followed by 40 cycles at 96 °C for 10 s and 63 °C for 1 min, and a final extension step at 63 °C for 5 min. The amplification product was analyzed by 0.8% agarose gel electrophoresis, with an expected band of 940 bp. The primary PCR product was diluted 1:50 with sterile water and used as template for secondary PCRs.

The secondary PCR was performed individually for each of the four *Plasmodium* species. Each secondary PCR was carried out in a 20 μl reaction containing 2 μl of the diluted primary PCR product, 0.4 μM of each primer (Fig. 1), 125 μM of each dNTPs, 2 mM of Mg^{2+}, and 0.5 unit of $Vent^{+}$ DNA polymerase (New England Biolabs Japan Inc., Tokyo, Japan). Cycling conditions consisted of an initial denaturation at 96 °C for 1 min, followed by 20 cycles at 96 °C for 10 s and 56 °C for 90 s, and a final extension at 56 °C for 5 min. Amplification products were analyzed by 2% agarose gel electrophoresis, with expected bands in the range of 87 to 233 bp.

Negative controls were included in both primary and secondary PCRs to monitor potential contamination.

2.5. cox3 PCR reproducibility and case detection

We used the Ambae samples (n = 37) to test the reproducibility of the primary PCR and its power to detect all *Plasmodium* infections. The primary PCR was repeated for all 37 samples without knowledge of the results of the initial attempt. For the repeat trial, all samples, including those that yielded no visible amplicons after the primary PCR, were subjected to nested PCR using the universal nested primers and species specific primers (Fig. 1).

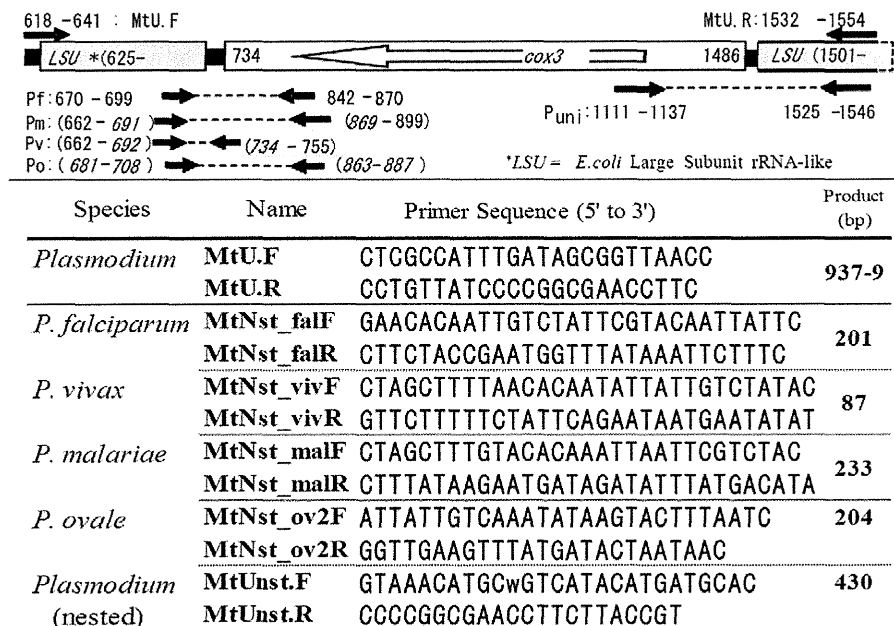
2.6. 18S rDNA and cytb amplifications

Amplificatons of the *Plasmodium* 18S rDNA were performed in the same reaction mixtures as described for the *cox3* amplifications, using primers and PCR conditions described previously [2].

Amplifications of the *Plasmodium cytb* [4] were performed with minor modifications. The primary PCR was performed in a 20 μl reaction mixture containing 6 μl of template DNA, 10 μl of the 2× Go Taq Green Master Mix (Promega KK, Tokyo, Japan), and 0.3 μM of each primer [4]. Cycling conditions consisted of an initial denaturation at 95 °C for 80 s, followed by 35 cycles at 95 °C for 40 s, 50 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min, which were identical to those described in [4] except for the denaturation temperature at 95 °C and the initial denaturation time of 2 min (80 s + 40 s of the initial cycle), as recommended by the manufacturer (Promega KK, Tokyo, Japan). The primary PCR product was diluted 1:50 with sterile water. The secondary PCR was performed separately for each pair of species–specific primers in a 20 μl reaction mixture containing 2 μl of the diluted product, 10 μl of the 2× Go Taq Green Master Mix, and 0.3 μM of each primer by using cycling conditions identical to those of the primary PCR, except that the number of amplification cycles was reduced to 20. Negative controls were included at all amplification steps.

2.7. Comparison of detection methods

Relative detection efficiencies of the different PCR methods were calculated as the ratios of positive cases for each *Plasmodium* species. To further evaluate the performance of the PCR methods, detection rates were compared at four parasitemia levels: 0, 1–3, 4–19, and ≥20



Species	Name	Primer Sequence (5' to 3')	Product (bp)
Plasmodium	MtU.F	CTCGCCATTTGATAGCGGTTAACC	937-9
	MtU.R	CCTGTTATCCCCGGCGAACCTTC	
P. falciparum	MtNst_falF	GAACACAATTGTCTATTCGTACAATTATTC	201
	MtNst_falR	CTTCTACCGAATGGTTTATAAATTCTTTC	
P. vivax	MtNst_vivF	CTAGCTTTTAACACAATATTATTGTCTATAC	87
	MtNst_vivR	GTTCTTTTTCTATTCAGAATAATGAATATAT	
P. malariae	MtNst_malF	CTAGCTTTGTACACAAATTAATTCGTCTAC	233
	MtNst_malR	CTTTATAAGAATGATAGATATTTATGACATA	
P. ovale	MtNst_ov2F	ATTATTGTCAAATATAAGTACTTTAATC	204
	MtNst_ov2R	GGTTGAAGTTTATGATACTAATAAC	
Plasmodium (nested)	MtUnst.F	GTAAACATGCwGTCATACATGATGCAC	430
	MtUnst.R	CCCCGGCGAACCTTCTTACCGT	

Fig. 1. Primer positions and sequences. Primers are represented by filled (black) arrows, and their nucleotide positions (np) are based on the *P. falciparum* mitochondrial genome sequence (GenBank accession number M76611). Numbers in parentheses are *P. falciparum*-corresponding positions for the other three species (approximate positions in italic). Nested PCR regions are indicated by dotted lines with primer directions represented by arrows. Fully nested universal genus-specific amplification using MtUnst.F (np 1111-1137) and MtUnst.R (np 1546-1525) are indicated as P_{uni}. Pf, Pm, Pv, and Po correspond to *P. falciparum*, *P. malariae*, *P. vivax*, and *P. ovale*, respectively. w = C/T in a primer sequence. The direction of transcription of the *cox3* gene is shown by the unfilled arrow.

parasites/200 leukocytes. The sensitivity and specificity of the *cox3* and the *cytb* PCRs were evaluated using the 18S rDNA PCR [2] as a standard. Sensitivity was determined as true positives/(true positives + false negatives), while specificity was determined as true negatives/(true negatives + false positives).

3. Results

3.1. cox3 PCR reproducibility and case detection

Two independent trials of the primary PCR were carried out to determine the reproducibility of our method. Of the 37 samples from Ambae, 27 (73.0%) were positive for both trials while two (5.4%) were positive in the first attempt but negative in the second attempt. These two samples that gave discordant results (numbers 17 and 23 in Table 1) were both microscopically negative. One sample (number 11) was negative for both *cox3* PCR trials (Table 1) but was positive for *P. vivax* by microscopy.

For the second trial, nested PCRs using universal nested primers and species specific primers were performed to determine the ability of the primary PCR to detect all *Plasmodium* infections. Neither nested PCRs produced additional positive cases when compared to the primary PCR. One sample (number 37) identified as *P. vivax*-positive by microscopy was detected as *P. falciparum*-positive by the *cox3* PCR instead (Table 1).

3.2. Overall detection efficiencies

Our *cox3* method showed a higher overall efficiency, detecting 21.3% more total species count (148 vs. 122) than the 18S rDNA PCR [2]. Higher relative efficiencies were observed for all four *Plasmodium* species, ranging from 1.10 for *P. falciparum* to 1.50 for *P. ovale*. Our *cox3* method was especially efficient in detecting cases of co-infections with two (relative efficiency of 1.72) or three (3.00) *Plasmodium* species (Table 2).

In contrast, the *cytb* method [4] showed a lower overall detection efficiency (0.56) than the 18S rDNA PCR in our field samples. Lower detection efficiencies were observed for *P. falciparum* (0.41), *P. vivax* (0.57), and mixed infection involving two species (0.56) (Table 2).

It is unclear why the *cytb* method failed to give results superior to the 18S rDNA method as described previously [4]. It is possible that the use of the GoTaq Green Master Mix (Promega KK, Tokyo, Japan) in our amplifications, which has a lower final dNTP concentration (0.2 mM each) than that described in [4] (2.5 mM each), might have a negative effect on amplification efficiencies.

3.3. Detection efficiencies at different parasitemia levels

Relative to the 18S rDNA PCR, our *cox3* PCR showed equal or higher overall efficiencies for all four species examined. Improvements in detection efficiencies were minor and not significant for samples with microscopic parasitemia (1.10–1.16). However, for microscopically negative samples, the improvement (1.54) was statistically significant (paired samples *t*-test; $p = 0.0074$), suggesting that our *cox3* PCR was better at detecting submicroscopic infections than the 18S rDNA PCR (Table 3).

The *cytb* PCR [4] has lower efficiency to detect *Plasmodium* infections than the 18S rDNA PCR at all parasitemia levels, with relative efficiencies ranging from 0.36 to 0.87. There was a general decrease in efficiency with decreasing parasitemia, especially for *P. falciparum* which accounted for the majority of infections in our samples (Table 3).

There were seven samples (samples 48, 56, 63, 65, 70, 84, and 89) in which parasites were observed under the microscope but yielded no amplicons in any of the three PCRs (Supplementary Table 1). In five of these samples (63, 65, 70, 84, and 89), the negative PCR results were also corroborated by the negative RDT results, suggesting reading errors by microscopists. One sample (56) was positive for *P. falciparum* by both microscopy and RDT but negative by all three PCRs, and one sample (48) was positive for *P. vivax* by microscopy but negative by PCRs. Both samples had low parasitemia, thus it was likely that parasite DNA was lost during extraction, leading to negative PCR results.

3.4. Sensitivity and specificity

We determined the sensitivity and specificity for the *cytb* and the *cox3* PCRs for each *Plasmodium* species, using the 18S rDNA PCR as a

Table 1
Reproducibility and detection efficiency of the *cox3* PCR.

Sample number	Microscope		*cox3*			
	Species	Parasitemia	Trial 1 Primary	Trial 2 Primary	Secondary (Genus)	Secondary (Species)
1	V	12	pos	pos	pos	V
2	neg	0	neg	neg	neg	neg
3	neg	0	pos	pos	pos	V
4	V	19	pos	pos	pos	V
5	F	9	pos	pos	pos	F
6	neg	0	pos	pos	pos	M
7	neg	0	pos	pos	pos	FV
8	V	2	pos	pos	pos	FV
9	neg	0	pos	pos	pos	FV
10	neg	0	pos	pos	pos	V
11	V	2	neg	neg	neg	neg
12	neg	0	pos	pos	pos	F
13	neg	0	pos	pos	pos	F
14	neg	0	neg	neg	neg	neg
15	neg	0	neg	neg	neg	neg
16	V	1	pos	pos	pos	V
17	neg	0	pos	neg	neg	neg
18	neg	0	neg	neg	neg	neg
19	V	67	pos	pos	pos	V
20	F	6	pos	pos	pos	F
21	F	60	pos	pos	pos	F
22	neg	0	neg	neg	neg	neg
23	neg	0	pos	neg	neg	neg
24	V	6	pos	pos	pos	V
25	neg	0	pos	pos	pos	F
26	neg	0	neg	neg	neg	neg
27	neg	0	neg	neg	neg	neg
28	V	8	pos	pos	pos	V
29	V	11	pos	pos	pos	V
30	neg	0	pos	pos	pos	FV
31	M	9	pos	pos	pos	M
32	V	15	pos	pos	pos	V
33	V	42	pos	pos	pos	V
34	F	22	pos	pos	pos	F
35	M	4	pos	pos	pos	M
36	F	16	pos	pos	pos	F
37	V	16	pos	pos	pos	F

Parasitemia was determined by counting the number of parasites against 100 leukocytes. The following notations are used: pos = positive, neg = negative, F = *P. falciparum*, M = *P. malariae*, and V = *P. vivax*.

standard. Our *cox3* PCR was able to detect every species identified by the 18S rDNA PCR at all parasitemia levels, thus giving sensitivities of 1. Except for *P. ovale*, the *cytb* PCR showed lower sensitivities than the *cox3* PCR at virtually all parasitemia levels. The *cytb* PCR also showed substantial sensitivity decline with decreasing parasitemia (Table 4).

Except for *P. ovale* which was rarely observed in our field samples, our *cox3* PCR showed lower overall specificities than the *cytb* PCR. At high parasitemia level (≥20 parasites/200 leukocytes), the specificities of both methods for each *Plasmodium* species were identical, whereas at lower parasitemia levels (<20 parasites/200 leukocytes), our *cox3* PCR showed lower specificities as a result of its ability to detect *Plasmodium* species not detected by the 18S method (Table 4).

Table 2
Efficiencies of the *cytb* and *cox3* methods compared to the 18S rDNA method.

	18S rDNA	*cytb*	Efficiency	*cox3*	Efficiency
P. falciparum	69	28	0.406	76	1.101
P. malariae	21	20	0.952	30	1.429
P. ovale	2	3	1.500	3	1.500
P. vivax	30	17	0.567	39	1.300
Total	122	68	0.557	148	1.213
2 species	18	10	0.556	31	1.722
3 species	1	1	1.000	3	3.000

Table 3
Efficiencies of the *cytb* and *cox3* methods at different parasitemia levels compared to the 18S rDNA method.

	Method	Parasitemia (parasites/200 leukocytes) 0	1–3	4–19	≥20	Overall
P. falciparum	*cytb*	0.25	0.29	0.38	0.85	0.41
	cox3	1.50	1.04	1.00	1.00	1.10
P. malariae	*cytb*	0.67	0.80	0.86	1.33	0.95
	cox3	1.67	1.80	1.14	1.33	1.43
P. ovale	*cytb*	N/D	1.00	1.00	N/D	1.50
	cox3	N/D	1.00	1.00	N/D	1.50
P. vivax	*cytb*	0.56	0.00	0.75	0.55	0.57
	cox3	1.56	1.00	1.50	1.00	1.30
All species	*cytb*	0.42	0.36	0.59	0.87	0.56
	cox3	1.54	1.14	1.16	1.10	1.21

N/D denotes not determined. At 0 and ≥20 parasites/200 leukocytes, no *P. ovale* was detected using the 18S rDNA method, thus the denominator was zero. For samples that were negative by microscopy (0), no *P. ovale* was detected by any method.

4. Discussion

We describe an improved PCR protocol whereby only one round of amplification was needed to reliably detect all malaria-positive individuals, including those with very low or undetectable parasitemia by conventional microscopy. Compared to the 18S rDNA PCR method [2], long considered the standard for PCR-based detection, our *cox3* PCR method showed both high sensitivities and greater efficiencies for all four *Plasmodium* species considered. The greater efficiencies may be attributed to the higher copy number of the mtDNA genome relative to the 18S rDNA in the parasites. For a subset of 16 samples, we diluted the DNA 1:10 and repeated the *cox3* PCR. We obtained the same number of total detected species as the 18S rDNA method using undiluted DNA (data not shown), consistent with the expectation based on the difference in copy numbers between these two targets.

We also tested a modified primer set for the primary PCR of the 18S rDNA method [5]. While they appeared to have a greater binding specificity, the number of *Plasmodium* species detected after the nested PCR were not improved when compared to the primers used in the original 18S rDNA method. Since the target of the modified primer set is about 1.6 times longer (about 1640 bp), and longer target fragments greatly reduce detection efficiency for older archival samples such as our samples from Vanuatu [14], we chose to use the original method [2] for comparison.

Suboptimal primer design might have contributed to the lower efficiencies of the *cytb* method, especially for the detection of *P. falciparum* and *P. vivax* (Table 3). For *P. falciparum*, the forward primer of the secondary PCR (PFCBF: 5′-ATTATTTATTGTATTATTTTTCTG-3′) has a very low G-C content (12.5%) and a low melting temperature of 40.8 °C (calculated by the nearest neighbor method of Oligo 1.5), which is substantially lower than the annealing temperature (50 °C) used in the PCR. For *P. vivax*, the forward primer of the secondary PCR (PVCBF: 5′-AGTTACCACAAGATATTTTT-3′) has a long weak-interacting segment of eight consecutive A or T at its 3′ end. Furthermore, a primer of the primary PCR (PCBR: 5′-CAGACCGTAAGGTTATAATTATGT-3′ [4], which is a reverse complement sequence of the reverse primer), has a self-annealing 8-nucleotide sequence (underlined) which may cause a primer dimer, since a rather low annealing temperature of 50 °C is used. These shortcomings might have compromised primer binding to template DNA and reduced the overall amplification and detection efficiencies.

Plasmodium knowlesi has recently been recognized to be an important human malaria species in Southeast Asia [13]. We have designed a *P. knowlesi* specific primer set that could be incorporated in our protocol. However the lack of *P. knowlesi* infection in our field samples prevented us to adequately test the efficiency and other performance parameters. Future studies using field samples with known *P. knowlesi* infections are currently being considered.

Table 4
Sensitivities and specificities of the *cytb* and *cox3* methods at different parasitemia levels compared to the 18S rDNA method.

		Parasitemia (parasites/200 leukocytes)									
		0		1–3		4–19		≥20		Overall	
		Sens*	Spec*	Sens	Spec	Sens	Spec	Sens	Spec	Sens	Spec
P. falciparum	*cytb*	0.08	0.96	0.29	1.00	0.38	1.00	0.85	1.00	0.38	0.98
	cox3	1.00	0.88	1.00	0.89	1.00	1.00	1.00	1.00	1.00	0.92
P. malariae	*cytb*	0.33	0.98	0.60	0.97	0.86	1.00	1.00	0.90	0.76	0.97
	cox3	1.00	0.97	1.00	0.88	1.00	0.95	1.00	0.90	1.00	0.93
P. ovale	*cytb*	N.D.	1.00	1.00	1.00	1.00	1.00	N.D.	0.96	1.00	0.99
	cox3	N.D.	1.00	1.00	1.00	1.00	1.00	N.D.	0.96	1.00	0.99
P. vivax	*cytb*	0.29	0.94	0.00	1.00	0.63	0.95	0.55	1.00	0.43	0.97
	cox3	1.00	0.91	1.00	1.00	1.00	0.79	1.00	1.00	1.00	0.93

No *P. ovale* was detected by the 18S rDNA method at parasitemia levels 0 and ≥20; sensitivities of the *cytb* and *cox3* methods were not determined (N.D.).
* Sens = Sensitivity, Spec = Specificity.

5. Conclusions

We described an improved PCR method that allows for less time- and resource-consuming detection of *Plasmodium* infections, while providing greater detection efficiencies than the 18S rDNA [2] and *cytb* methods [4], especially for submicroscopic infections. Our method will contribute to improved surveillance in national malaria elimination programs, especially in low-endemicity settings [15].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.parint.2014.09.006.

Acknowledgements

The authors would like to express their sincere gratitude to the people of Vanuatu and Kenya for their help in the surveys. This work was supported by the Japan Society for Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (KAKENHI Nos. 24390141 and 26257504), the Core-to-Core Program B, Asia–Africa Science Platforms, the Swedish Research Council grants (523-2009-3233, 348-2012-6346 and 348-2013-6311) and the Health Labor Sciences Research Grant, Research on Global Health Issues (PI: A.K.).

References

[1] Bousema T, Drakeley C. Epidemiology and infectivity of *Plasmodium falciparum* and *Plasmodium vivax* gametocytes in relation to malaria control and elimination. Clin Microbiol Rev 2011;24:377–410.

[2] Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN. 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 1993;58:283–92.

[3] Swarthout TD, Counihan H, Senga RK, van den Broek I. Paracheck-Pf accuracy and recently treated *Plasmodium falciparum* infections: is there a risk of over-diagnosis? Malar J 2007;6:58.

[4] Putaporntip C, Buppan P, Jongwutiwes S. Improved performance with saliva and urine as alternative DNA sources for malaria diagnosis by mitochondrial DNA-based PCR assays. Clin Microbiol Infect 2011;17:1484–91.

[5] Singh B, Bobogare A, Cox-Singh J, Snounou G, Abdullah MS, Rahman HA. A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. Am J Trop Med Hyg 1999;60:687–92.

[6] Kimura M, Kaneko O, Liu Q, Zhou M, Kawamoto F, Wataya Y, et al. Identification of the four species of human malaria parasites by nested PCR that targets variant sequences in the small subunit rRNA gene. Parasitol Int 1997;46:91–5.

[7] Vaidya AB, Arasu P. Tandemly arranged gene clusters of malarial parasites that are highly conserved and transcribed. Mol Biochem Parasitol 1987;22:249–57.

[8] Preiser PR, Wilson RJM, Moore PW, McCready S, Hajibagheri MAN, Blight KJ, et al. Recombination associated with replication of malarial mitochondrial DNA. EMBO J 1996;15:684–93.

[9] Wilson RJ, Williamson DH. Extrachromosomal DNA in the Apicomplexa. Microbiol Mol Biol Rev 1997;61:1–16.

[10] Hikosaka K, Watanabe Y, Kobayashi F, Waki S, Kita K, Tanabe K. Highly conserved gene arrangement of the mitochondrial genomes of 23 *Plasmodium* species. Parasitol Int 2011;60(2):175–80.

[11] Bourgeois N, Boutet A, Bousquet PJ, Basset D, Douard-Enault C, Charachon S, et al. Comparison of three real-time PCR methods with blood smears and rapid diagnostic test in *Plasmodium* sp. infection. Clin Microbiol Infect 2010;16:1305–11.

[12] Haanshuus CG, Mohn SC, Mørch K, Langeland N, Blomberg B, Hanevik K. A novel, single-amplification PCR targeting mitochondrial genome highly sensitive and specific in diagnosing malaria among returned travelers in Bergen, Norway. Malar J 2013;12:26.

[13] Singh B, Lee KS, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J, et al. A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. Lancet 2004;363:1017–24.

[14] Hwang J, Jaroensuk J, Leimanis ML, Russell B, McGready R, Day N, et al. Long-term storage limits PCR-based analyses of malaria parasites in archival dried blood spots. Malar J 2012;11:339.

[15] Harris I, Sharrock WW, Bain LM, Gray KA, Bobogare A, Boaz L, et al. A large proportion of asymptomatic *Plasmodium* infections with low and sub-microscopic parasite densities in the low transmission setting of Temotu Province, Solomon Islands: challenges for malaria diagnostics in an elimination setting. Malar J 2010;9:254.

MALARIA
JOURNAL

RESEARCH Open Access

Determinants of the use of insecticide-treated bed nets on islands of pre- and post-malaria elimination: an application of the health belief model in Vanuatu

Noriko Watanabe[1*], Akira Kaneko[1,2], Sam Yamar[3], Hope Leodoro[3], George Taleo[3], Takeo Tanihata[4], J Koji Lum[5] and Peter S Larson[6,7]

Abstract

Background: Insecticide-treated nets (ITNs) are an integral piece of any malaria elimination strategy, but compliance remains a challenge and determinants of use vary by location and context. The Health Belief Model (HBM) is a tool to explore perceptions and beliefs about malaria and ITN use. Insights from the model can be used to increase coverage to control malaria transmission in island contexts.

Methods: A mixed methods study consisting of a questionnaire and interviews was carried out in July 2012 on two islands of Vanuatu: Ambae Island where malaria transmission continues to occur at low levels, and Aneityum Island, where an elimination programme initiated in 1991 has halted transmission for several years.

Results: For most HBM constructs, no significant difference was found in the findings between the two islands: the fear of malaria (99%), severity of malaria (55%), malaria-prevention benefits of ITN use (79%) and willingness to use ITNs (93%). ITN use the previous night on Aneityum (73%) was higher than that on Ambae (68%) though not statistically significant. Results from interviews and group discussions showed that participants on Ambae tended to believe that risk was low due to the perceived absence of malaria, while participants on Aneityum believed that they were still at risk despite the long absence of malaria. On both islands, seasonal variation in perceived risk, thermal discomfort, costs of replacing nets, a lack of money, a lack of nets, nets in poor condition and the inconvenience of hanging had negative influences, while free mass distribution with awareness campaigns and the malaria-prevention benefits had positive influences on ITN use.

Conclusions: The results on Ambae highlight the challenges of motivating communities to engage in elimination efforts when transmission continues to occur, while the results from Aneityum suggest the possibility of continued compliance to malaria elimination efforts given the threat of resurgence. Where a high degree of community engagement is possible, malaria elimination programmes may prove successful.

Keywords: Malaria, Insecticide-treated net (ITN), The Health Belief Model (HBM), Motivation, Sustainability, Malaria elimination, Islands, Aneityum, Ambae

* Correspondence: n881052@gmail.com
[1]Department of Parasitology, Osaka City University Graduate School of Medicine, Osaka, Japan
Full list of author information is available at the end of the article

© 2014 Watanabe et al.; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

Background

An estimated 3.3 million lives have been saved since 2000 as a result of a major scale-up of vector control interventions, including high coverage of insecticide-treated nets (ITNs) through combined catch-up (mass, free distribution of ITNs) and keep-up (long-term, routine access to new ITNs) strategies despite the global funding gap [1,2].

Various factors are associated with the use and non-use of ITNs in malaria endemic areas [3-13]. Free and comprehensive ITN distribution programs may successfully increase the level of ITN ownership and encourage high levels of coverage compensating for a lack of household or administrative financial resources [3-5,10-13]. However, resource challenged and undeveloped infrastructure is associated with decreased likelihood of both ITN ownership and use even when ITNs are possessed [6,8,9,13]. Free ITN programs may induce a community wide expectation of free ITNs though some programs have been criticized for enabling the diversion of ITNs for uses other than malaria control in extremely poor settings [6,9,12]. Common factors that may predict consistent ITN use in Papua New Guinea [11], Solomon Islands [3,12] and Vanuatu [4] are knowledge [3,4,11,12], malaria risk perception [3,4,11], social life (activities, sleeping place and hanging space) [3,4,11] and ITN accessibility, sufficiency, price, physical condition, maintenance, replacement, effectiveness and insecticide [3,4,11,12]. Common factors that may cause variation in ITN use and compliance are seasonal factors such as heat, mosquito density and variable levels of transmission [3,4,11,12].

This paper aims to investigate the perceptions and beliefs about malaria and the use of ITNs in Vanuatu (a country on the verge of malaria elimination [14]). Vanuatu is an archipelago of 83 islands located in the southwest Pacific. The vast majority of ni-Vanuatu, as the indigenous population is known, live in rural areas and engage almost wholly in subsistence farming [15]. *Plasmodium falciparum* and *Plasmodium vivax* transmission persist throughout the majority of Vanuatu's islands, while *Plasmodium malariae* transmission rarely occurs [1,16]. Vanuatu has two seasons: the cold, dry season from May to October, and a hot, wet (rainy) season from November to April. *Plasmodium falciparum* incidence shows marked seasonality, whereas *P. vivax* incidence shows less marked seasonal patterns [16-18]. In 2008, Vanuatu formally declared a national goal of eliminating malaria by 2020 using a spatially progressive strategy with significant financial support being made available mainly through the Global Fund to fight AIDS, Tuberculosis and Malaria [14,19]. In cooperation with efforts to eliminate lymphatic filariasis, a national ITN programme has resulted in a sharp decline in malaria cases [17,20]. The Malaria Indicator Survey (MIS) in 2011 indicated that 71.9% of surveyed people slept under an ITN the previous night, given that the household owned at least one ITN. The MIS also found that Vanuatu households owned an average of 1.99 ITNs per household [19]. Likely as a result, the annual parasite incidence (API) decreased from 73 per 1,000 population in 2003 to nine per 1,000 population in 2011 [21]. As the burden of malaria decreases, it will be important to understand the perceptions of preventive measures to sustain gains in the control and eventual elimination of disease [22].

As ITN programmes depend on the acceptance and active involvement of individuals and communities, human behavioural and social factors will influence ITN use [13]. The Health Belief Model (HBM), a framework commonly used to explore compliance to health interventions [23-25] including community-based interventions [24] can be used to interpret perceptions and net-use behaviours as was shown in previous studies in Tanzania [5,7]. The HBM has six constructs to explain and predict preventive health behaviours: modifying factors, perceived threat (severity and susceptibility), benefits, barriers, self-efficacy and cues to action [23-25]. In this study, the HBM framework was used to explore and predict health behaviours (consistent ITN use) in the context of reduced malaria risk.

Methods

(i) Study settings and design

The study areas were purposefully selected to contrast a region where malaria transmission continues to occur with an island that has sustained elimination for several years. Ambae Island, an area of low and sporadic malaria transmission, is located in Penama Province. Aneityum Island, where a community-based, elimination-specific effort since 1991 has successfully halted malaria incidence, is located in Tafea Province in the south [26-28] (Figure 1).

Ambae

Transmission of *P. falciparum, P. vivax* and *P. malariae* in the area is considered meso-endemic [16]. Indigenous malaria transmission persists, although the prevalence rate of malaria has been declining in recent years (Kaneko *et al.,* unpublished data). Ambae island (405 sq km) has a population of 10,407 (2009 Vanuatu National Census). The study was conducted at Lolovoli village in northeast Ambae with a population of over 200.

Aneityum

Transmission of *P. falciparum and P. vivax* in the area was considered hypo- to meso-endemic before the introduction of an elimination-specific intervention [16,26]. In 1991, weekly mass drug administration (MDA) with chloroquine, pyrimethamine/sulphadoxine and primaquine to the entire population (718 islanders) was carried out on Aneityum Island for nine weeks [26,27]. Simultaneously, ITNs were provided to the entire population, free of charge to mothers and children less than five years of age, and at a cost of US$4 (real price) to adults and US$2 to