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.

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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

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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.

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

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http://dx.doi.org/10.1016/j.parint.2014.09.006
1383-5769/

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).

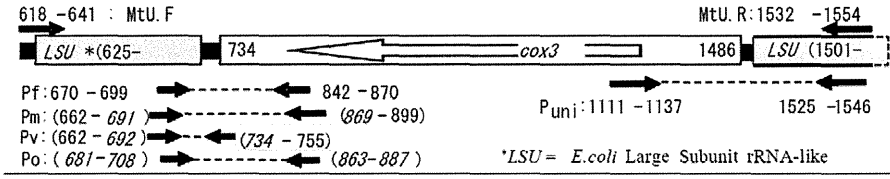
2.6. 18S rDNA and cytb amplifications

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	**MtUnst.F**	GTAAACATGCwGTCATACATGATGCAC	**430**
(nested)	**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*			
			Trial 1	Trial 2		
	Species	Parasitemia	Primary	Primary	Secondary	
					(Genus)	(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.).

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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

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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

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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

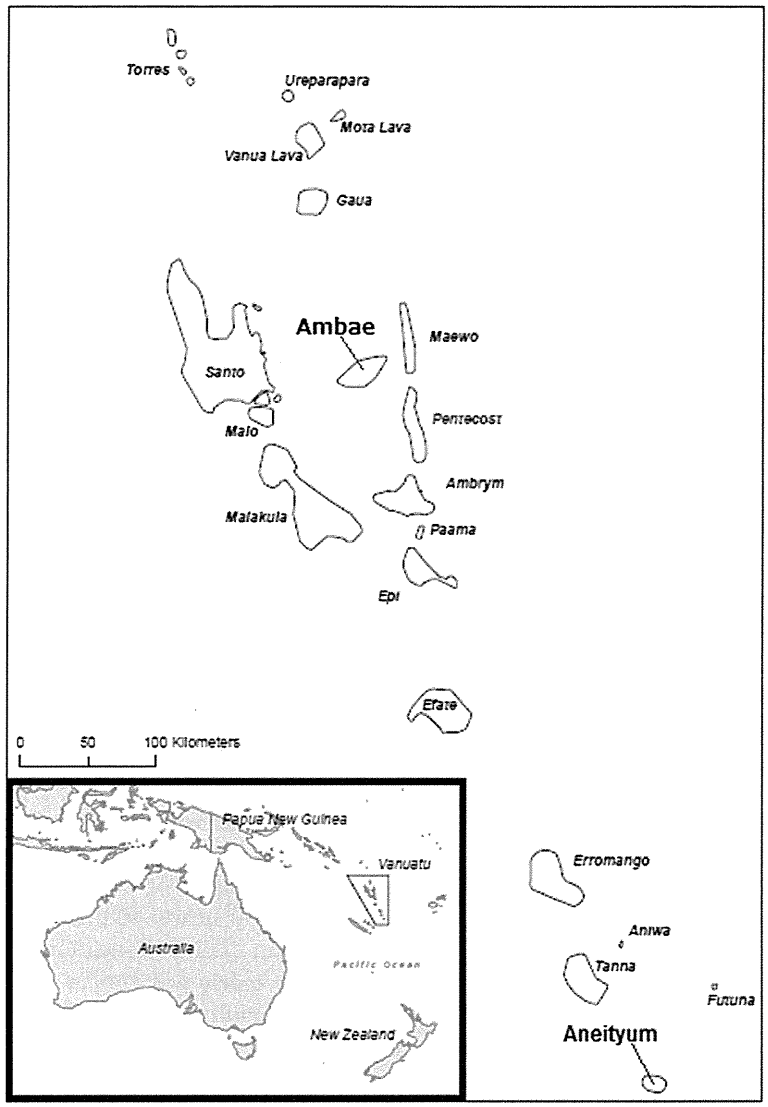

Figure 1 Locations of study areas on Vanuatu.

schoolchildren [26,27]. In addition, larvivorous fish were introduced into breeding sites of *Anopheles farauti* [26,27]. After the MDA, *P. falciparum* immediately disappeared, while *P. vivax* disappeared from 1996, with the exception of two instances of imported infections (one mixed infection in 1993 and one *P. vivax* infection in 1999) [26,27]. In 2002, a small outbreak of *P. vivax* was reported mainly among children born after 1991 [27,28]. After the age-selected MDA using chloroquine and primaquine, the outbreak quickly subsided except for a few asymptomatic infections of *P. vivax* [27].

Aneityum Island has a total land area of 159.2 sq km with a population of 915 (2009 Vanuatu National Census). Inyeug has a small airport and grass runway. Tourism is the main source of income. There are three main villages: Analgaut, Port Patrick and Unmet [26,27]. The study was conducted at the largest village, Analgaut, due to its population size and ease of access.

(ii) Data collection procedures

Data were collected on the two islands in July 2012. Residents were notified ahead of time by local leaders and health workers. Local leaders called a community meeting and explained the intent and process of the survey. At that time, community members were asked to take part in the survey and requested to arrive at the dispensary or community meeting place on scheduled dates.

Knowledge, attitudes and practices (KAP) survey

The team conducted a cross-sectional survey (knowledge, attitudes and practices) on the two islands. A questionnaire (see Additional file 1) translated into Bislama was

administered by local survey assistants fluent in local languages and known to the community and who had been hired and trained to interview respondents. Responses for very young children were provided by an accompanying parent or adult household representative. A questionnaire was structured to capture some of the HBM constructs (perceived severity, benefits and self-efficacy) and action (ITN use the previous night). Potential answers (reasons) for non-use such as absence of mosquitoes/rain, low risk of infection (not being afraid of contracting malaria), excessive heat, inconvenience of hanging nets, nets in poor condition and a lack of nets in the home were provided in a questionnaire.

Interviews (FGDs, KIIs and IDIs)

For data triangulation, focus group discussions (FGDs), key informant interviews (KIIs) and in-depth interviews (IDIs) were used. A researcher with local facilitators, conducted all discussion and interviews on both islands. Focus group discussants were recruited by local facilitators. Key informants such as *kastomary* chiefs (customary chiefs), teachers, religious leaders, health committee members, health care workers, and shop sellers were purposefully selected. In-depth interviewees were recruited through convenience sampling to ensure a breadth and depth of insights.

Semi-structured interview questions (see Additional file 2) were pilot tested on a few informants to review the answers and assess the relevance to the different HBM constructs. All FGDs, KIIs and IDIs were recorded using notes and a digital device in the presence of local facilitators. The questions were asked in English by a researcher, and then translated into Bislama by local facilitators. The participants' answers were directly translated from local language to English by local facilitators. Where this was not necessary, English was used as a common language. Each interview transcript was shared and discussed among local facilitators and key informants to explain results and obtain feedback.

(iii) Data management and analysis

Analysis of the KAP survey instrument (statistical methods)

Differences between the two islands were analysed using standard Chi-square tests for categorical variables. T-tests and Wilcoxon tests were used to compare continuous measures between islands. All statistical analyses were performed using R version 2.15.1 (CRAN 2012).

Analysis of interviews

The HBM was used as the theoretical framework, where six main HBM constructs (i.e., severity of malaria, susceptibility to malaria, benefits of ITN use, barriers to ITN use, cues to ITN use and self-efficacy) served as the pre-existing categories. The theory-based analysis style (a deductive approach) was applied [29,30]. First, a categorization matrix was developed [30]. Each interview transcript was read multiple times to identify meaning units, which were condensed, coded and assigned to the pre-existing HBM categories in a matrix [30,31].

Parallel analysis in a mixed methods study

This study employed parallel analysis in a mixed methods study [32,33]. Data collection and analysis were carried out separately and the findings were not compared or consolidated until the interpretation stage [33]. Qualitative and quantitative results were used to complement each other [29,33].

Ethical considerations

This research was approved by the Vanuatu Ministry of Health, and the Institutional Review Board of State University of New York, Binghamton (#1578-10). Written and verbal consent was obtained before starting the survey and interviews. All respondents were assured that their responses would remain confidential.

Results

KAP survey

The result from the KAP survey instrument used on Ambae and Aneityum islands are presented in Table 1. A total of 91 of nearly 200 residents participated in the survey in the village of Lolovoli on Ambae Island, compared to 354 of nearly 400 residents of Analgaut village on Aneityum Island. Educational attainment of adults (aged 18 or older) was similar in both islands. The vast majority of adults had completed only primary education, with half completing secondary school (modifying factors). About half of the residents of Ambae, but only one quarter of Aneityum (48.8 *vs* 25.3%, P <0.0001), reported having been diagnosed with malaria by a health professional in the past (modifying factors). Significantly more children under the age of five on Ambae reported having had malaria in the past than on Aneityum (42.9 *vs* 7.9%).

No significant difference was found between the two islands in terms of questions which might be applicable to the HBM constructs (perceived severity, benefits, and self-efficacy) and action. ITN use the previous night on Aneityum (73.0%) was higher than that on Ambae (68.2%) although not significant. Nearly all survey respondents claimed to be afraid of malaria (severity). Half stated that malaria was a deadly disease (severity). Seventy-nine per cent of survey respondents reported that they considered ITNs to be an effective means of malaria prevention (the malaria-prevention benefits). 93% of respondents reported liking to sleep under an ITN (self-efficacy).

Table 1 Knowledge, attitudes and practices (KAP) survey results

HBM constructs	Questions	All	Ambae	Aneityum	P
	N	445	91	354	
	Under 5 years old	53	6	47	
	5 to 17 years old	215	34	181	
	18 to 30 years old	69	15	54	
	Over 30 years old	108	36	72	
	Age range	0-75	0-75	0-74	
	Female	221	49	172	
Action	Used last night	72.0%	68.2%	73.0%	0.44
Modifying factors					
Educational attainment (aged 18 or older)	None	0.6%	0.0%	0.8%	0.22
	Primary	41.2%	45.1%	39.7%	
	Secondary	48.6%	47.1%	49.2%	
	Unknown	9.6%	7.8%	10.3%	
Malaria history	I had malaria	30.1%	48.8%	25.3%	<.0001
By age	Under 5 years old	13.3%	42.9%	7.9%	
	5 to 17 years old	20.1%	35.7%	17.5%	
	18 to 30 years old	32.8%	50.0%	27.7%	
	Over 30 years old	54.2%	60.0%	51.4%	
Individual beliefs					
Perceived severity	I am afraid of malaria (disease)	98.7%	100%	98.4%	1
	Malaria is deadly	55.1%	55.3%	55.1%	1
Perceived benefits	Malaria-prevention	79.4%	78.8%	79.6%	1
Self-efficacy	I am willing to sleep under a net	92.8%	95.3%	92.2%	0.4

The reasons given for a lack of ITN use are shown in Figure 2. Although the malaria history of respondents under the age of five suggested indigenous malaria transmission on Ambae during recent years (Table 1), low malaria risk perception was the most common barrier to compliance (Figure 2). On Aneityum, perceived low mosquito density with excessive heat acted as barriers to ITN use during the dry season (Figure 2). On both islands, a lack of nets in the home, poor condition and the inconvenience of hanging were common barriers (Figure 2).

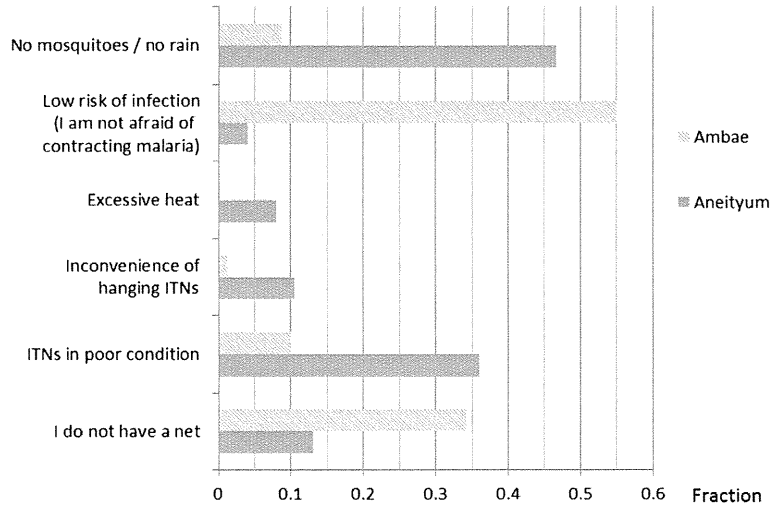

Figure 2 Reasons for non-use of insecticide-treated bed nets based on knowledge, attitudes and practices (KAP) survey.

Interviews

A total of 35 residents of Lolovoli village on Ambae Island took part in five FGDs, seven KIIs and eight IDIs, while a total of 57 residents of Analgaut on Aneityum took part in six FGDs, ten KIIs and 17 IDIs on Aneityum (Table 2). All results are presented in Figure 3.

Action

Many participants on both islands reported using ITNs. A few key informants on both islands stated that they had screens on windows. A few households on both islands reported not owning an ITN. Some participants on both islands noted that their ITNs had multiple holes. A few participants on both islands noted that they used their ITNs despite having multiple holes. A few participants on both islands reported alternative use.

"A few people recycled old nets. Pieces were used for various purposes." (Male FGD, Ambae)

"Five years ago, I saw a child using nets for fishing. Now, health committee encourages people not to use nets for farming or fishing." (Female KII, Ambae)

"I saw some children using nets for fishing this year." (Male KII, Aneityum)

A male key informant on Aneityum expressed his concern over the ecological and safe disposal methods for used and worn-out ITNs.

Modifying factors

A few participants on both islands noted that they did not know how to read and write. All participants were aware that mosquito bites are associated with malaria. Some participants on both islands stated that they had financial difficulty in paying school expenses for several children. *Kastom* (traditional) medicine was used on both islands. A few adults from both islands who reported having had malaria in the past stated that they used ITNs. Some participants on both islands reported forming proactive health behaviours.

"Some children use nets. They are used to sleeping under a net." (Female KII, Ambae)

"I use a net every day. I am used to sleeping under a net, because I try to prevent malaria." (Male IDI, Ambae)

"I use a net. Everyone uses a net. We feel strange, if we do not use nets." (Female IDI, Aneityum)

Participants on Ambae were reported difficulties in consistently using ITNs, while participants on Aneityum reported being highly motivated to use them. On Ambae, many participants stated that the disappearance of malaria reduced the perceived need for sleeping under ITNs. In this context, some male discussants and key informants expressed difficulties in sustaining malaria control efforts in the village.

"It is very difficult to sustain efforts, because of the absence of malaria. Some people do not use nets. After the net distribution, malaria is not endemic." (Male FGD, Ambae)

"Malaria is disappearing. Some people no longer need nets. Raising awareness is necessary, but a community radio has been broken. Additional funding will be needed to repair a radio. This area faces critical shortage of nurses. The dispensary and hospital are too far. This is a completely neglected area. We need more funds." (Male KII, Ambae)

In contrast, an effort to promote healthy life and to eliminate malaria on Aneityum motivated many individuals to use ITNs. Most people reportedly engaged in elimination efforts.

"We are happy to live on this island. We try to maintain a healthy environment. We sleep under a net and keep our village clean." (Female FGD, Aneityum)

"Malaria depopulated this island in the past. Now we should increase the population. Many people use nets to live a healthier life." (Male KII, Aneityum)

Perceived threat of malaria (perceived severity of and susceptibility to malaria)

Participants on Ambae were likely to state that some villagers did not use ITNs because they were not concerned

Table 2 Demographics of interviews

	Ambae		
	N	**Age (years old)**	**Female**
FGD	20 (5 groups)	13-44	65.0%
KII	7	28-64	20.0%
IDI	8	20-52	40.0%
ALL	35	13-64	54.3%
	Aneityum		
	N	**Age (years old)**	**Female**
FGD	30 (6 groups)	16-31	66.7%
KII	10	21-65	50.0%
IDI	17	16-67	52.9%
ALL	57	16-67	59.6%

FGD: Focus Group Discussion.
KII: Key Informant Interview.
IDI: In-Depth Interview.

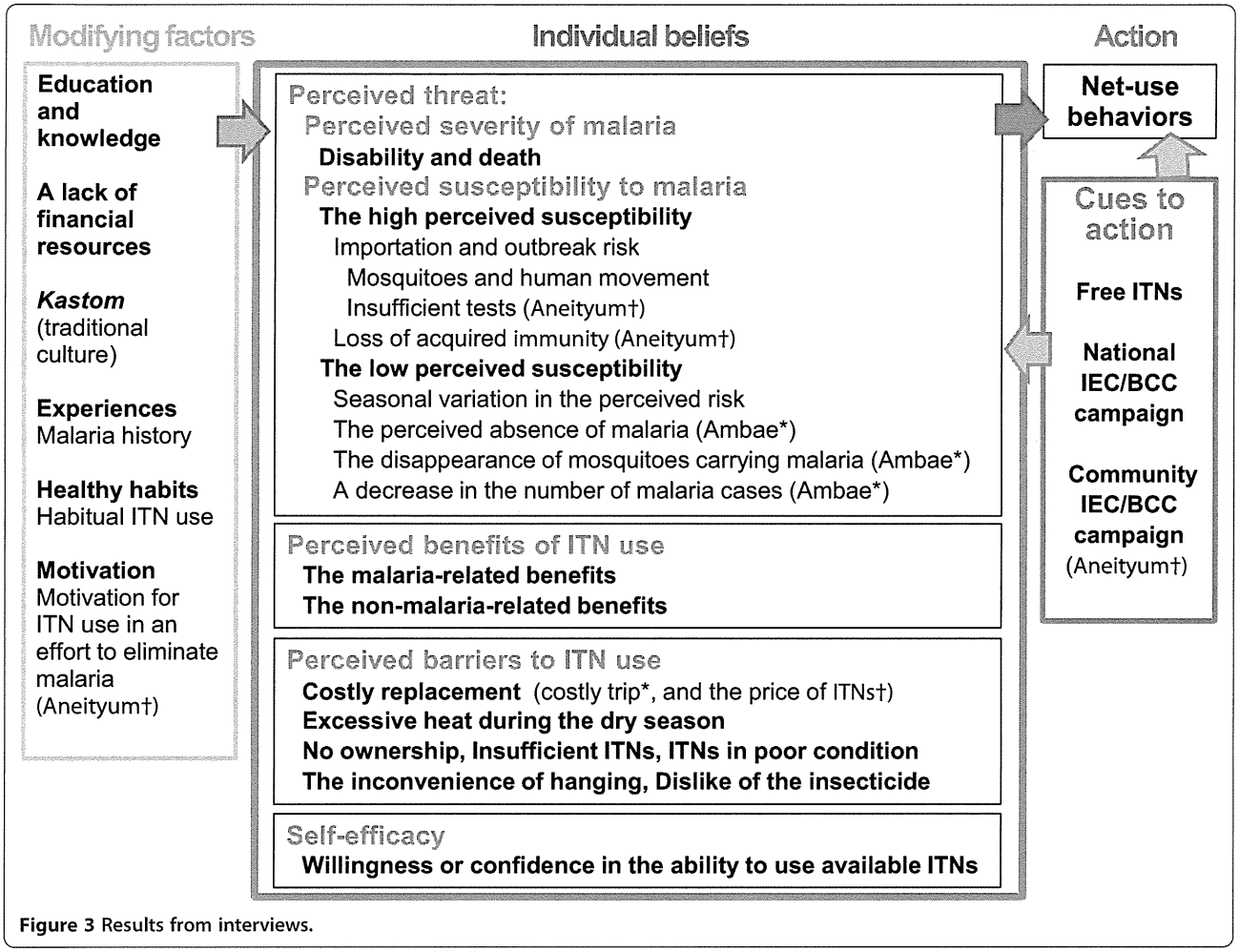

Figure 3 Results from interviews.

about the possibility of contracting malaria, while participants on Aneityum stated that using ITNs was necessary out of fear that accidental importation could cause an outbreak of malaria on the island.

Malaria was not recognized as a matter of community concern requiring community action on Ambae. A few youth interviewees stated that visitors from the malarious villages would drive parasite importation, while many participants believed that they were not at risk for malaria infection. Beliefs about the disappearance of malaria transmitting mosquitoes and the reduction in malaria cases were cited as major reasons for decreased concerns of risk.

"Last year, Lolowai hospital malaria team came and killed mosquito larvae. Now we are not bothered by malaria-infected mosquitoes. All 14 patients with fever had negative RDT results in this year. Men were not willing to come to the aid post at the onset of fever. No new case of filariasis, dengue fever, or malaria has occurred. But some people keep on using nets, because they are afraid of malaria, and they are used to sleeping under a net." (Female KII, Ambae)

"We live in a relatively cold climate on the island. The cold weather reduces mosquito populations. Other villages have more mosquitoes. People catch malaria at South Ambae or North Ambae, but no one has malaria in this village, because anopheline mosquitoes are not found in this village. So some people do not use nets. I am usually bitten 20 times per day by mosquitoes such as Culex and Aedes. A few cases of malaria have been reported in recent years. Malaria is not a serious problem in this village. But malaria remains a serious problem in Sakao." (Male IDI, Ambae)

In contrast, malaria was widely perceived as a serious disease and recognized as a matter of community concern requiring community action on Aneityum. Except for a few participants who did not perceive themselves to be at risk for malaria infection, most participants on Aneityum believed malaria posed a very real threat.

"We want live in a healthy environment. We fear that imported malaria will increase. Because only one person takes blood on this island." (Female KII, Aneityum)

"I am afraid of malaria. Malaria will come back again. So, I think I need a net." (Male IDI, Aneityum)

Young people were viewed as more susceptible to malaria due to loss of acquired immunity by some participants on Aneityum.

"Older people would notice the signs of malaria. But younger people would not notice. Their bodies do not learn how to deal with malaria. We used to have malaria here, but we do not have malaria now. I do not know what will happen in the future." (Female KII, Aneityum)

Community members on Aneityum were very concerned that both humans and mosquitoes constituted and continuing threat to maintaining malaria elimination.

"A big swamp has been managed by committee members. Most people use nets every day." (Female KII, Aneiyum)

"The only problem on this island is that malaria-infected people stay without being tested." (Male KII, Aneityum)

"Malaria-carrying mosquitoes will kill us. I use a net." (Female IDI, Aneityum)

"Because Aneityum is a place of quarantine in Vanuatu, many yachts visit this island. Some foreigners walk around the village without the testing for a few weeks. Local boat passengers are not screened. I am worried that I may have contracted malaria in Tanna, because we (fishers) travel back and forth. All people entering Aneityum are not screened. Air passengers have been screened, but cruise passengers have not been screened this year. An airplane and a ship are problematic." (Male IDI, Aneityum)

Perceived benefits of ITN use

The most cited benefit of ITN use on both islands was the prevention of malaria. The protection from mosquito bites was the other most commonly cited benefit on both islands.

"Malaria-infected anopheline mosquitoes are not found in this village, however people still use nets, because people protect themselves from mosquito bites. I am bitten all the time." (Female KII, Ambae)

"I appreciate the benefits of the insecticide, because the treated nets provide very good protection from being bitten by mosquitoes. I use a net every day." (Male KII, Aneityum)

Protection against other diseases (lymphatic filariasis on Ambae, and scabies on Aneityum) as well as against pests (cockroaches, fleas, flies, and head lice on Aneityum), and keeping warm in cold weather on Ambae were very occasionally reported as the non-malaria-related benefits of ITN use. A few key informants on Aneityum noted that mass use of ITNs eliminated scabies.

Perceived barriers to ITN use and maintenance

On both islands, the most commonly reported barrier to ITN use was that the ITNs were uncomfortable to sleep under during very hot conditions. Many participants reported that they slept without ITNs during the dry season when temperatures are very hot and winds quite weak.

"When the weather is hot during the dry season, we do not use nets." (Male KII, Ambae)

"If people feel uncomfortable during the dry season, people enjoy sleeping outside and use mosquito coils. If we do not see mosquitoes outside, we do not use coils. During the rainy season, we use nets." (Female KII, Aneityum)

Dislike of the insecticide was identified as barriers on both islands by a limited number of participants.

"I do not use a net, because I use kastom medicine. My grandmother is a kastom healer. But my family members go to the dispensary at the onset of fever. My mother encourages me to go to the dispensary, but I do not want to go." (Female IDI, Ambae)

"I do not like the chemical smell. Babies suck a net. But I use a net, because I know it works for protection." (Female KII, Aneityum)

A female discussant on Aneityum noted that she had never slept under an ITN, because she believed that ITNs caused suffocation. A few participants on both islands reported the inconvenience of hanging rectangular ITNs and a preference for conical ITNs.

High costs were reported as barriers to replacement of old and worn out ITNs. Distance and accessibility to ITN distribution points (the cost of travelling to and from hospital in North Ambae) reportedly acted as hurdles to obtaining new ITNs on Ambae. A key informant noted that Lolowai hospital did not allow the aid post to deliver ITNs. The price of ITNs (almost US$5) on Aneityum was noted among the majority of female participants. The issue of user charges was initially raised within the female

FGDs. A few discussants and interviewees reported having no intention to purchase in spite of their beliefs about their chances of getting malaria and the malaria-prevention benefits of ITN use. A female interviewee on Aneityum who reportedly did not buy an ITN for her baby stated that she would be willing to get free ITNs. Another female interviewee on Aneityum noted that she sold a portion of her crops or woven baskets to purchase a new ITN.

Self-efficacy and cues to action

Most participants on both islands stated that they were confident in their ability to use available ITNs. Receipt of a free ITN was commonly seen as beneficial on both islands.

> *"In the past, nets were not easily available for every person. Now nets are distributed free of charge. Everyone has a net. That is why people keep and use." (Male KII, Ambae)*

> *"In the past, rich people could afford to buy nets, but now everybody has a treated net. That is good." (Female KII, Aneityum)*

A few key informants on Ambae stated that high community coverage would contribute to a reduction in malaria transmission.

All participants on both islands recognized the sources of malaria information (cues) such as service delivery points (the dispensary on Aneityum and hospital on Ambae), health staff, the provincial malaria team, public notice, a community meeting, family meeting, health talk, church activities, school activities and Radio Vanuatu. Some male discussants and key informants on Ambae reported that they suffered lack of funds to sustain government-led efforts against a disappearing disease, while most participants on Aneityum intended to utilize available resources to prevent the re-introduction of malaria. Community-based elimination activities such as surveillance, vector control measures and awareness campaigns reportedly encouraged consistent ITN use on Aneityum.

KAP survey and interviews

Results from the KAP survey (Figure 2) and interviews (Figure 3) showed that the HBM constructs which negatively influenced ITN use were the low perceived susceptibility to malaria (a reduction in malaria risk) and barriers to ITN use (Table 3). The low perceived susceptibility had more impact on Ambae than Aneityum (Table 3). Results from interviews (FGDs, KIIs and IDIs) revealed the motivation and healthy habits were less influenced by the low perceived susceptibility and perceived barriers.

This study indicated that three determinants of ITN use influenced net-use behaviours: (1) malaria risk, (2) intervention services (tools and services), and (3) personal factors (modifying factors and self-efficacy) (Table 4). These factors were not independent, but rather were interlinked. Seasonal variation in the perceived risk of malaria influenced utilization of and compliance to malaria interventions and attitudes. Interventions influenced the knowledge, attitudes and practices. A lack of resources such as money, time and knowledge occasionally acted as a brake, while motivational beliefs sustained intervention and malaria risk.

Discussion

Malaria risk perception (mainly due to perceived susceptibility), free ITNs (cues to action), community-based intervention services (cues to action) and personal factors (modifying factors) encouraged individuals on Aneityum to maintain the use of ITNs, in spite of material and psychological costs. On Ambae, the low perceived susceptibility along with material and psychological costs was associated with reduced compliance to ITNs.

Table 3 Determinants of non-use

Low perceived susceptibility					**Perceived barriers**				
Beliefs	**Islands**		**QL**	**QT**	**Beliefs**	**Islands**		**QL**	**QT**
Dry season	Am	An	QL	QT	Excessive heat	Am	An	QL	
							An		QT
Perceived low mosquito density	Am	An	QL	QT	Dislike of the insecticide	Am	An	QL	
Low risk of infection	Am	An	QL	QT	Inconvenience of hanging nets	Am	An	QL	QT
Perceived absence of malaria	Am		OL		Nets in poor condition	Am	An	QL	QT
Disappearance of mosquitoes carrying malaria	Am		QL		A lack of nets in the home	Am	An	QL	QT
					Costly services				
					Time and cost to access	Am	An	QL	
Reductions in the numbers of malaria cases	Am		QL		User charge		An	QL	

QL: Qualitative results (FGDs, KIIs and IDIs), QT: Quantitative results (KAP survey).
Am: Ambae, An: Aneityum.

Table 4 Determinants of access and use

Determinants	Beliefs or factors	HBM constructs	Islands	
Malaria risk				
Beliefs or factors associated with ITN access and use	Health consequences (death)	Severity	Am	An
	Social consequences (depopulation)	Severity		An
	Loss of acquired immunity	Susceptibility		An
	Human movement	Susceptibility	Am	An
	Insufficient screening	Susceptibility		An
	Hot, wet (rainy) season	Susceptibility	Am	An
	High mosquito density	Susceptibility	Am	An
	The potential risks of malaria infections	Susceptibility	Am	An
Beliefs or factors associated with non-use	Cold, dry season	Susceptibility	Am	An
	Low mosquito density	Susceptibility	Am	An
	Low risk of infection	Susceptibility	Am	An
	The disappearance of malaria-infected anopheline mosquitoes	Susceptibility	Am	
	The reduction in malaria incidence	Susceptibility	Am	
Intervention services (tools and services)				
Beliefs or factors associated with ITN access and use	Free mass distribution (catch-up)	Cues	Am	An
	National campaigns	Cues	Am	An
	Community-based campaigns	Cues		An
	ITN prevention (malaria)	Benefits	Am	An
	ITN protection (mosquitoes)	Benefits	Am	An
	ITN prevention (other diseases)	Benefits	Am	An
	ITN protection (other pests)	Benefits		An
Beliefs or factors associated with non-use	Time and cost to replace nets (keep-up)	Barriers	Am	An
	Insufficient or a lack of ITNs in the home	Barriers	Am	An
	ITNs in poor condition	Barriers	Am	An
	Inconvenience of hanging	Barriers	Am	An
	Excessive heat in the net (discomfort)	Barriers	Am	An
	Side effects of the chemical	Barriers	Am	An
Personal factors (modifying factors and self-efficacy)				
Beliefs or factors associated with access and use	Willingness or confidence to use ITNs	Self-efficacy	Am	An
	Knowledge	Modifying factors	Am	An
	Experiences (malaria history)	Modifying factors	Am	An
	Healthy habits (consistent ITN use)	Modifying factors	Am	An
	Motivation for ITN use in an effort to eliminate malaria (healthy life)	Modifying factors		An
Beliefs or factors associated with non-use	Unwillingness to buy or use ITNs	Self-efficacy	Am	An
	The absence of financial resources	Modifying factors	Am	An
	Insufficient knowledge	Modifying factors	Am	An

Am: Ambae, An: Aneityum.

Perceptions of disease severity and the potential risks of malaria infections (importation and outbreak risk [14]) were linked to sustained use of ITNs on Aneityum. However, the perceived absence of malaria was linked to non-use of ITNs on Ambae in spite of a low but still present risk of malaria transmission, suggesting a potentially detrimental effect on sustained ITN coverage and use in the context of disappearing disease. Perceived low mosquito density during the dry season, hampered consistent use of ITNs even on Aneityum. This outcome agrees with previous results on seasonality that may predict variation in ITN use [3-5,7,8,10-13]. Thermal discomfort during the hot, wet season was not mentioned as a barrier to ITN use on both islands, indicating that

the perceived higher risk of malaria outweighs the perceived discomfort of being hot during the wet (rainy) season, consistent with a previous study in Zanzibar, Tanzania [5]. Participants did, however, that ITNs were uncomfortable during the dry season, a tendency which could compromise malaria control efforts where malaria transmission still occurs. Psychological, physical or financial burden of services were directly linked to non-use of ITNs on both islands in spite of the malaria-prevention and non-malaria prevention benefits of ITN use. Routine access to new nets (keep-up strategies) had negative influences on ITN ownership on both islands despite free mass distribution (catch-up strategies) and campaigns (cues to actions). Expanding access to keep-up nets may improve coverage and use [2]. As cost significantly dampens demand and decreases access to health services among the poor at greater risk, ITNs should be provided via the public sector as a public good, like vaccines with a generous donation, because malaria is linked to poverty [34-37].

Results from focus group discussions, key informant interviews, and in-depth interviews showed that a lack of resources, discomfort, dislike, unwillingness and insufficient knowledge had negative influences on individual decision making regarding ITN use on both islands, while knowledge of preventive measures against importation and outbreak risk, healthy habits and the motivation had positive influence on decision making on Aneityum. Although a previous study in Burkina Faso has shown that the motivation for the use of new ITNs can decrease within a year due to inhabitants' conception of malaria and the inconvenience of using ITNs [38], this study showed that the sustained motivation for ITN use in an effort to promote healthy life and to eliminate malaria influenced net-use behaviours on Aneityum, indicating that sustained motivation continuously increases knowledge, encourages healthy habits and creates demand for ITNs regardless of circumstances (local culture, a hot, humid tropical climate, low risk infection, costly services in resource-poor settings and poverty) behind net-use behaviours. This study implies the primary factors that predict high ITN coverage and use on Aneityum are individual and collective motivations and engagement in elimination activities. Unlike the one-off vaccination campaigns, ITN programmes require a high degree of participation and practices to be sustained over time [39]. Community-based campaigns including information, education and communication (IEC)/behavioural change communication (BCC) minimize risk behaviours on Aneityum, where a participatory process maintains high ITN coverage and use at the community level toward malaria elimination. The findings from Aneityum provide clues to sustainable ITN use and malaria elimination in areas of reduced malaria transmission.

Limitations

The HBM was employed to explore perceptions and beliefs about malaria and ITN use. This study focused on individuals' beliefs and net-use behaviours without fully taking into account social, economic and emotional factors that may also influence preventive health behaviours. Time was limited and the number of interviews that was feasibly performed was small. In particular, the results from interviews (FGDs, KIIs and IDIs) are limited to be generalized to the wider population of each island. As the survey and interviews were performed during the dry season, the results may have differed during the wet season when mosquitoes are abundant. Some responses relating to ITN use may be subject to social desirability bias. Finally, there may be a certain degree of loss of nuances and depth as a result of the direct translation from Bislama to English in conducting interviews (FGDs, KIIs and IDIs).

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.

Additional files

Additional file 1: A questionnaire.
Additional file 2: Interview questions.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NW designed the survey. AK, JKL, GT, SY and HL coordinated the field work. JKL, PSL, SY, HL, TT and NW conducted KAP survey. PSL conducted analysis of the KAP survey instrument. NW conducted interviews (FGDs, KIIs and IDIs) with SY and HL. NW, SY, HL conducted analysis of interviews (FGDs, KII and IDIs) with support from PSL and AK. Parallel analysis and manuscript drafting was carried out by NW with contributions from PSL and AK. All authors read and approved the final manuscript.

Acknowledgements

The authors wish to express their gratitude to the residents of the survey communities. The authors thank the local facilitators, particularly Alan, Jennifer, Doris, Roselyn (Lolovoli village, Ambae Island), Simon, Nelson, Akissie, Natu and Rennie (Analgaut village, Aneityum Island). The authors are grateful to Dr Ros Seyha (WHO) and Dr Mitsuru Fukui (Osaka City University) for their valuable comments. This work (PI: AK) was supported by Swedish Research Council grants (523-2009-3233, 348-2012-6346, and 348-2013-6311), Japan Society for Promotion of Science (JSPS) Core-to-Core Program B, Asia-Africa Science Platforms. JSPS KAKENHI grant Numbers (24390141 and 26257504), and Health Labor Sciences Research Grant, Research on Global Health Issues.

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Received: 18 September 2014 Accepted: 25 October 2014
Published: 20 November 2014

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doi:10.1186/1475-2875-13-441
Cite this article as: Watanabe *et al.*: 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. *Malaria Journal* 2014 **13**:441.