Cross-sectional study of asymptomatic malaria and seroepidemiological surveillance of seven districts in Gia Lai province, Vietnam

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Abstract

Background: Malaria elimination by 2030 is an aim of many countries in the Greater Mekong Sub-region, including Vietnam. However, to achieve this goal and accelerate towards malaria elimination, countries need to determine the extent and prevalence of asymptomatic malaria as a potential reservoir for malaria transmission and the intensity of malaria transmission. The purpose of this study was to determine the prevalence of asymptomatic malaria and seropositivity rate in several districts of Gia Lai province in the Central Highlands of Vietnam.

Methods: A cross-sectional survey of asymptomatic malaria and serological testing was conducted in 3283 people living at 14 communes across seven districts in Gia Lai province in December 2016 to January 2017. Finger prick capillary blood samples were tested for malaria using rapid diagnostic testing and polymerase chain reaction (PCR), as well as detecting antibodies against 3 Plasmodium falciparum and 4 Plasmodium vivax antigens by indirect enzyme-linked immunosorbent assay (ELISA). Age-seroprevalence curves were fitted using reverse catalytic models with maximum likelihood.

Results: The study population was predominantly male (65.9%, 2165/3283), adults (88.7%, 2911/3283) and of a minority ethnicity (72.2%, 2371/3283), with most participants being farmers and outdoor government workers (90.2%, 2960/3283). Using a small volume of blood (≈ 10 µL) the PCR assay revealed that 1.74% (57/3283) of the participants had asymptomatic malaria (P. falciparum 1.07%, P. vivax 0.40%, Plasmodium malariae 0.15% and mixed infections 0.12%). In contrast, the annual malaria prevalence rates for clinical malaria in the communities where the participants lived were 0.12% (108/90,395) in 2016 and 0.22% (201/93,184) in 2017. Seropositivity for at least one P. falciparum or one P. vivax antigen was 38.5% (1257/3262) and 31.1% (1022/3282), respectively. Age-dependent trends in the proportion of seropositive individuals in five of the districts discriminated the three districts with sustained low malaria prevalence from the two districts with higher transmission.

Conclusions: Asymptomatic Plasmodium carriers were found to be substantially more prevalent than clinical cases in seven districts of Gia Lai province, and a third of the population had serological evidence of previous malaria.

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Background

Despite continued decreases in the number of malaria cases outside of Africa, there are still more than 200,000 cases reported annually across Asia and the Pacific, primarily in 11 endemic countries (Cambodia, China, the Lao People’s Democratic Republic, Myanmar, Malaysia, Papua New Guinea, the Philippines, the Republic of Korea, Solomon Islands, Vanuatu and Vietnam) [1]. Countries across the Greater Mekong Subregion (GMS) have pledged to eliminate all *Plasmodium* species of malaria by 2030 [2]. There is lasting value to eliminating malaria as nearly all countries that have achieved malaria elimination have been able to maintain this status despite the presence of competent vectors, imported cases and proximity to malaria endemic countries [3]. Additionally, malaria elimination is critical to combating drug resistant malaria as nearly all countries that have achieved malaria elimination strategies for Vietnam.

**Keywords**: Asymptomatic malaria, *Plasmodium falciparum*, *Plasmodium vivax*, Gia Lai province, Vietnam, PCR, Serology, Drug resistance

*(NMCPs) still rely almost exclusively on blood film microscopy or rapid diagnostic tests (RDTs) for the routine detection of malaria and case investigations at communal health stations and for large scale malaria surveys. Although these tools are relatively inexpensive and widely available, they lack the sensitivity to detect infections in individuals who are asymptomatic and/or carry sub-microscopic parasite densities. These infections need to be identified by more sensitive molecular tools, such as polymerase chain reaction (PCR), to aid NMCPs develop elimination strategies and target resources to areas of residual malaria transmission.

As transmission decreases, parasitological surveys designed to identify ongoing infections will require large sample sizes, becoming logistically and economically unfeasible for NMCPs. Other malaria transmission metrics such as entomological inoculation rate also have inherent limitations in low transmission areas due to very low numbers of infected mosquitoes [11–13]. Alternatively, serological markers of parasite exposure have been proposed as a potentially cost-effective proxy for malaria transmission intensity in low endemic areas [10, 11, 14, 15]. Seropositivity to parasite antigens in a specific population is assumed to represent cumulative exposure to past malaria infections thus reducing the probability of missing exposure events, including asymptomatic infections. Moreover, by analysing age-seroprevalence patterns it is possible to identify historical trends in transmission intensity in a specific population group and how these trends differ between communities [11, 16, 17]. Such serological tools are of special interest in pre-elimination scenarios existing in Vietnam, with marked spatial transmission heterogeneity (i.e. hotspots of transmission surrounded by areas of no transmission), where geographical stratification in transmission levels becomes essential to target NMCP interventions efficiently [18].

The primary objective of this cross-sectional study was to characterize the parasite reservoir of *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* in an asymptomatic population working and residing in Gia Lai province, Vietnam. Three endpoints were assessed to characterize malaria burden in the study population: (1) prevalence of parasite positive individuals determined by PCR, (2) prevalence of individuals with a history of malaria exposure measured by detection of antibodies to *P. falciparum* or *P. vivax*, and (3) proportion of *P. falciparum* parasitemia.
*P. falciparum* parasites with molecular markers associated with drug resistance.

**Methods**

**Study location**

The study was conducted between December 2016 and January 2017 at located in seven different districts of Gia Lai province. Gia Lai is located in the Central Highlands of Vietnam on the border with Cambodia, has a population of approximately 1.3 million people residing in an area of 15,500 km² distributed in 17 districts and city divisions (Fig. 1). Malaria transmission is perennial, typically rising after the start of the rains in May and peaking between September and December, with the lowest incidence from February to April. Delayed *P. falciparum* malaria clearance following artesinin-based combination therapy (*i.e.* dihydroartemisinin-piperaquine, DHA-PPQ) was reported in Gia Lai province in 2010 and 2017 [19, 20].

Cross-sectional malaria surveys were done in 14 military-civilian health facilities across the seven districts: Chu Prong (la Mo and la Puch communes), Chu Puh (la Hla, la Le and la Phang communes), Chu Se (la Hlop and la Ko communes), Duc Co (la Dom, la Nan and la Pnon communes), la Grai (la Chia and la O communes), Kong Chro (So Ro commune), and Krong Pa (Chu RCam commune) (Fig. 1). Subjects residing and/or working in these areas include both military personnel and civilians, who regularly receive primary health care at military medical facilities. Chu Pong, Duc Co and la Grai districts are located close to the border with Cambodia, where individuals are mainly responsible of border security (Fig. 1). Chu Se and Chu Puh are located in the centre of the province, with a predominance of soldiers working in farming. Finally, Kong Chro and Krong Pa districts are in the east, with predominance of local civilian population.

**Sample size**

The asymptomatic malaria prevalence was assumed to be greater than 1.25% based on a malaria survey conducted in Krong Pa district in April 2016, in which three malaria cases were detected by blood film microscopy out of 240 people screened (Dr. Ro Mah Huan, Center for Disease Control and Prevention, Gia Lai province, unpublished data). It was determined that a minimum of 3,052 participants would be required to detect a PCR positive rate of ≥1.25% with a two-sided 95% confidence interval no wider than 1% with alpha and beta errors of 0.05 and 0.20, respectively. Allowing for 7% to 8% of subjects withdrawing their consent to participate in the study, it was decided to recruit at least 3,265 to 3,296 participants.

Volunteer recruitment and enrolment

Both asymptomatic civilians and government workers from the Vietnam People’s Army eligible for medical care in the area covered by the selected military medical facilities were non-randomly invited to participate in the study. The inclusion criteria for the study were: (1) working or residing in Gia Lai province during the 14 days prior to the study visit, (2) 5 to 65 years of age, and (3) willingness to provide information (*e.g.* occupation, recent travel history, health check) and a finger prick blood sample. The exclusion criteria were: (1) being unwilling to provide consent, information or finger prick capillary blood sample, (2) inability to communicate well with the study staff, and (3) presenting with tympanic body temperature > 38 °C or reporting other malaria symptoms such as headache; these individuals were referred to the nearest health care facility. Following informed consent, study staff collected finger prick capillary blood samples for RDT (SD Bioline Malaria Ag Pf/Pv, Standard Diagnostics Inc., Abbott, Princeton, NJ, USA) and four filter paper blood spot samples were collected on 3MM filter paper (Whatman, Buckinghamshire, UK) for PCR analysis and antibody detection. Blood spots were stored individually in zip-closure plastic bags with desiccant (10 silica gel beads). Body temperature was measured, and demographic information was collected.

**Detection of malaria by PCR**

DNA was extracted from filter paper blood spots (approximately 10 µL of whole blood) using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) as per manufacturer’s protocol and eluted in 150 µL of AE buffer. Five µL was used as template in a single round multiplex PCR targeting small subunit (SSU) ribosomal RNA for simultaneous detection of *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*, as previously described [21]. PCR products were analysed by gel electrophoresis on 3% Tris–Acetate EDTA gel and compared to those produced by positive controls. Sensitivity of this method for detection of *P. falciparum* was tested using standards prepared from *P. falciparum* D6 laboratory line (Sierra-Leone, Africa). Briefly, highly synchronous ring-stage parasite cultures at 5% parasitaemia were centrifuged, washed twice in 100% plasma and resuspended at 50% haematocrit. These suspensions were dispensed into 50 µL aliquots in LoBind Eppendorf tubes and stored at -80°C. The parasite DNA was extracted from 50 µL aliquots and serially diluted. Using these standards, the lower limit of detection (LOD) for PCR was ≥ 0.25 parasites/µL. The PCR performance acceptance threshold was set tenfold greater than LOD at ≥ 2.5 parasites/µL (equivalent to 4.16 parasites in a
Fig. 1  Map of Gia Lai province, Vietnam. The study districts highlighted are in blue: Ia Grai (1), Duc Co (2), Chu Prong (3), Chu Se (4), Chu Puh (5), Kon Chro (6) and Krong Pa (7)
PCR reaction or 0.833 parasites/µL DNA eluate) with respective positive DNA controls included into every batch of PCR analysis. As DNA extraction efficiency control, filter paper spots containing 50 parasites per spot were prepared using the synchronous ring-stage D6 parasites and included in every batch of DNA extraction and subsequent PCR. When testing field samples, positive PCR results were accepted (i.e. bands on the gel of the size corresponding to the respective positive controls) if they were reproduced in at least 2 out of 3 PCR conducted. In addition, detection of *P. knowlesi* was carried out as previously described by Imwong et al. [22].

**Molecular markers of drug resistance**

Prevalence of artemisinin-resistance validated mutations in the *P. falciparum* kelch-13 gene (WHO report on artemisinin and ACT resistance, August 2018, [23]; *i.e.* F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H and C580Y) were evaluated by sequencing of the 850 bp *Pfkelch*-13 gene region covering amino acid 427 to 687, using a previously described method [24]. For markers of piperaquine resistance, mutation in *exo-E415G* (exonuclease gene PF3D7_1362500) and copy number of *plasmeisin* 2 and 3 were analysed as previously described [25, 26]. In addition, mutations in the *P. falciparum* chloroquine resistance transporter (*Pfcrt*) gene at codons 72 to 76 associated with chloroquine resistance were analysed by PCR amplification and sequencing, as previously described [27]. Copy number of *P. falciparum* multidrug resistance protein 1 (*Pfmdr1*) gene, which is associated with lumefantrine and mefloquine resistance, was determined by qPCR using SYBR Green chemistry [28]. Copy numbers > 1.5 was considered a gene duplication.

**Serological tests**

Antibodies against *P. falciparum* and *P. vivax* were detected by indirect enzyme-linked immunosorbent assay (ELISA). For antibody elution, two 5 mm discs were punched from dried blood spots, placed in a 96-well plate, and mixed with 1,120 μL of elution buffer containing PBS/0.05% Tween20/0.05% NaN3. Plates were incubated overnight at ambient temperature (~23 °C) and with shaking (200 rpm), and pink/red colored eluates were stored at 4 ºC. The following recombinant proteins were produced: *PfAMA-1* (3D7 strain), *PfMSP1* (19 kD C-terminal fragment, 3D7), *PfCSP* (3D7), *PvAMA-1* (Palo Alto), *PvMSP1* (19 kD C-terminal fragment), *PvCSP* allelic variant 210 (Sal-1) and *PvCSP* allelic variant 247 (PNG_M69059.1). All proteins were produced at Burnet Institute (Melbourne, Australia), except *PfMSP1* and *PvMSP1* produced at Dalian Institute of Biotechnology (Dalian, China).

ELISA was then used to measure IgG recognition of recombinant proteins. Briefly, 96-well microplates (Corning, Corning, NY, USA) were coated with 25 ng/well of antigen in PBS or carbonate buffer, sealed and incubated overnight at 4 °C. Total IgG from human serum (Sigma-Aldrich, St. Louis, MS, USA) were used as plate standards and directly coated in control wells at three different dilutions (50 ng, 25 ng and 12.5 ng). Plates were washed and blocked with 1% skimmed milk powder in PBS/0.05% Tween20 for 2 h. Fifty μL of antibody eluates were tested in duplicate at 1/200 dilution together with positive (confirmed malaria infected patients by light microscopy) and a negative control (unexposed Vietnamese). After incubation at room temperature for 2 h, peroxidase-conjugated goat anti-human IgG secondary antibody (Sigma-Aldrich) was added for 1 h, washed and mixed with 50 μL of 2,2’-azino-di-(3-ethylbenzthiazoline sulfonic acid (Sigma-Aldrich). Reaction was stopped after 15 min with 50 μL of 2 M H2SO4, and optical density (OD) values read at 405 nm in a Multiskan™ FC Microplate Photometer (ThermoFisher, Waltham, MA, USA). Duplicates with > 25% variation were repeated once, and those with > 25% variation after repetition were excluded from analysis. The mean OD from blank wells was subtracted from samples, controls and IgG standards at each plate, and total IgG standards were then used to normalize mean sample OD between plates. Fifteen percent of the study samples (n = 575) were retested for all seven antigens to assess reproducibility of serological assays, resulting in pairwise Pearson’s correlation coefficients ranging 0.877–0.952 (all < 5% Bonferroni-adjusted significance level, Additional file 1: Figure S1). The cut-off for ELISA seropositivity against each antigen was defined as the mean OD of negative controls plus three times the standard deviation.

**Definitions and statistical analysis**

The main dependent variable used this study was malaria infection based on PCR analysis. Independent demographic variables included age (stratified in seven age groups), ethnicity (Kinh versus ethnic minorities), sex, occupation (farmer, student, and government worker) and district.

Individuals were considered exposed to *P. falciparum* or *P. vivax* if they tested seropositive for at least one of the species antigens. Age-seroprevalence curves were fitted using reverse catalytic conversion models with maximum likelihood methods in R (R Core Team, v3.5.1), as previously described [17]. The model calculates one seroconversion rate (λ, *i.e.* the rate at which seronegative individuals become seropositive per year) and one seroreversion rate (ρ, *i.e.* the rate at which seropositive individuals become seronegative per year). Seroconversion rate can
thus be used as a measure of malaria force of infection or transmission intensity, whereas seroreversion rates can be interpreted as a measure of persistence of the antibody response [11]. Differences in demographic characteristics and malaria prevalence by PCR or serological data were determined using Pearson’s chi-squared (χ²) tests with Bonferroni corrections, or Fisher’s exact test when sample size was small. Risk factor analysis for *P. falciparum* and *P. vivax* seropositivity was conducted by running univariate logistic regression models for age, sex, ethnic group, occupation, district of residence and PCR positivity. Statistical analyses were performed in IBM SPSS 22.0 or Stata Version 12.0 (StataCorp, TX, USA), and graphs were created in Prism v8.0 (GraphPad, CA, USA). A significance level of *p* < 0.05 was defined for all tests.

**Results**

**Characteristics of the study population**

A total of 3,283 individuals consented to participate and were enrolled in the study from health facilities in Chu Prong (n = 330), Chu Puh (n = 600), Chu Se (n = 400), Duc Co (n = 530), Ia Grai (n = 220), Kong Chro (n = 600) and Krong Pa (n = 603) districts. Overall, the participants were predominantly adults (≥ 18 years of age; 88.7%, 2911/3283), male (65.9%, 2165/3283), and belonging to an ethnic minority group (72.2%, 2371/3283), with most participants engaged in farming and outdoor work activities (90.2%, 2960/3283) (Table 1). However, demographic characteristics between districts were heterogeneous: participants enrolled in Chu Puh and Chu Se districts were exclusively adult male government workers engaged in production labor in rubber and coffee farms (n = 1000), whereas participants from Kong Chro and Krong Pa were almost exclusively from local ethnic minority groups with a high proportion of individuals under 20 years of age (≥ 30.9%; other districts: ≤ 22.3%).

The annual number of clinical malaria cases reported by the 14 communal health stations by microscopy and/or RDT between 2014 and 2017 are presented in Table 2 (detailed data for each district are presented in the Additional file 1: Table S1). Malaria prevalence (all species) declined from 0.77% (626/81,346) in 2014 to 0.12% (108/90,395) in 2016, but increased to 0.22% (201/93,184) in 2017 [29]. During this period, the average proportion of cases attributable to *P. falciparum* was 58.8% (range 38.9–73.6%), whereas *P. vivax* accounted for 39.7% (24.9–59.2%).

### Table 1: Study population demographic characteristics at the seven districts in Gia Lai province, Vietnam

| By district | Total | p-value* |
|-------------|-------|----------|
| | N % | Chu Puh n % | Chu Se n % | Chu Prong n % | Duc Co n % | Ia Grai n % | Krong Pa n % | Kong Chro n % |
| Participants | 3283 100 | 600 18.3 | 400 12.2 | 330 10.1 | 530 16.1 | 220 6.7 | 603 18.4 | 600 18.3 |
| Age group | | | | | | | | |
| 5–9y | 244 6.8 | 0 0.0 | 0 0.0 | 35 10.6 | 30 5.7 | 20 9.1 | 65 10.8 | 74 12.3 |
| 10–19y | 528 16.1 | 134 22.3 | 44 11.0 | 18 5.5 | 52 9.8 | 10 4.5 | 121 20.1 | 149 24.8 |
| 20–29y | 1381 42.1 | 411 68.5 | 354 88.5 | 76 23.0 | 212 40.0 | 55 25.0 | 133 22.1 | 140 23.3 |
| 30–39y | 475 14.5 | 45 7.5 | 1 0.3 | 84 25.5 | 75 14.2 | 38 17.3 | 120 19.9 | 112 18.7 |
| 40–49y | 287 8.7 | 10 1.7 | 1 0.3 | 50 15.2 | 60 11.3 | 48 21.8 | 65 10.8 | 53 8.8 |
| 50–59y | 211 6.4 | 0 0.0 | 0 0.0 | 27 8.2 | 69 13.0 | 22 10.0 | 59 9.8 | 34 5.7 |
| 60–65y | 177 5.4 | 0 0.0 | 0 0.0 | 40 12.1 | 32 6.0 | 27 12.3 | 40 6.6 | 38 6.3 |
| Sex | | | | | | | | |
| Male | 2165 65.9 | 599 99.8 | 400 1000 | 190 57.6 | 346 65.3 | 113 51.4 | 221 36.7 | 296 49.3 |
| Female | 1118 34.1 | 0 0.0 | 0 0.0 | 140 42.4 | 184 34.7 | 107 48.6 | 382 63.3 | 304 50.7 |
| Ethnicity | | | | | | | | |
| Kinh | 912 27.8 | 303 50.5 | 201 50.3 | 162 49.1 | 197 37.2 | 19 8.6 | 1 0.2 | 29 4.8 |
| Ethnic minority | 2371 72.2 | 297 49.5 | 199 49.8 | 168 50.9 | 333 62.8 | 201 91.4 | 602 99.8 | 571 95.2 |
| Occupation | | | | | | | | |
| Farmer | 1737 52.9 | 0 0.0 | 0 0.0 | 197 59.7 | 393 74.2 | 181 82.3 | 475 78.8 | 491 81.8 |
| Student | 323 9.8 | 0 0.0 | 0 0.0 | 37 11.2 | 33 6.2 | 21 9.5 | 123 20.4 | 109 18.2 |
| Gov. worker | 1223 37.3 | 600 100.0 | 400 100.0 | 96 29.1 | 104 19.6 | 18 8.2 | 5 0.8 | 0 0.0 |

* Chi square test
Prevalence of asymptomatic malaria

Two individuals tested positive for malaria by RDT (0.06%, 2/3283). The prevalence of asymptomatic malaria detected by PCR was 1.74% (57/3283; *P. falciparum* 1.07%, *P. vivax* 0.40%, *P. malariae* 0.15%, and mixed infections 0.12%; Table 3). Excluding Chu Puh and Chu Se, which had a different demographic composition and little to no clinical malaria cases, the prevalence of asymptomatic malaria for the other five districts was 2.1% (49/2283). Asymptomatic individuals were detected in all districts except Chu Se district (Table 3). *P. falciparum* was the predominant species (61.4%, 35/57) followed by *P. vivax* (22.8%, 13/57). The difference in total and individual *Plasmodium* species malaria distribution was significantly different between the seven districts surveyed. *P. ovale* and *P. knowlesi* malaria were not detected.

| Year | Total | Prevalence by district (%) | p-value* |
|------|-------|-----------------------------|----------|
|      | Population n | % | Chu Puh | Chu Se | Chu Prong | Duc Co | Ia Grai | Krong Pa | Kong Chro |
| a) Population prevalence of clinical malaria | | | | | | | | | |
| 2014 | 81,346 | 626 (0.77) | 0.09 | 0.07 | 0.53 | 0.73 | 0.55 | 3.94 | 3.78 |
| 2015 | 85,114 | 300 (0.35) | 0.04 | 0.02 | 0.28 | 0.36 | 0.10 | 2.08 | 1.72 |
| 2016 | 90,395 | 108 (0.12) | 0.02 | 0.00 | 0.08 | 0.14 | 0.02 | 0.45 | 0.98 |
| 2017 | 93,184 | 201 (0.22) | 0.03 | 0.00 | 0.31 | 0.58 | 0.27 | 0.27 | 0.18 |
| b) Population prevalence of *P. falciparum* cases | | | | | | | | | |
| 2014 | 81,346 | 404 (0.50) | 0.06 | 0.03 | 0.15 | 0.47 | 0.34 | 2.44 | 2.88 |
| 2015 | 85,114 | 174 (0.20) | 0.03 | 0.02 | 0.23 | 0.08 | 0.06 | 1.33 | 1.24 |
| 2016 | 90,395 | 42 (0.05) | 0.02 | 0.00 | 0.04 | 0.02 | 0.00 | 0.18 | 0.51 |
| 2017 | 93,184 | 148 (0.16) | 0.01 | 0.00 | 0.20 | 0.49 | 0.14 | 0.16 | 0.10 |
| c) Population prevalence of *P. vivax* cases | | | | | | | | | |
| 2014 | 81,346 | 214 (0.26) | 0.02 | 0.04 | 0.38 | 0.26 | 0.20 | 1.46 | 0.79 |
| 2015 | 85,114 | 122 (0.14) | 0.01 | 0.00 | 0.05 | 0.29 | 0.03 | 0.72 | 0.45 |
| 2016 | 90,395 | 64 (0.07) | 0.00 | 0.00 | 0.04 | 0.13 | 0.02 | 0.27 | 0.44 |
| 2017 | 93,184 | 50 (0.05) | 0.01 | 0.00 | 0.11 | 0.08 | 0.13 | 0.10 | 0.03 |

Data provided by Center for Disease Control and Prevention for Gia Lai province, Vietnam [29].

Clinical malaria was defined as symptomatic cases reported by communal health stations with a confirmed microscopy and/or RDT result.

Table 3 Prevalence of malaria and *Plasmodium* species by PCR in the study area, stratified by seven districts in Gia Lai province, Vietnam

| Total | By district | p-value* |
|-------|-------------|----------|
| Samples tested | 3,283 | | |
| PCR result, n (%) | | | |
| Negative | 3,226 (98.3) | 592 (98.7) | 400 (100) | 327 (99.1) | 506 (95.5) | 219 (99.6) | 588 (97.5) | 594 (99) | p < 0.001 |
| Positive | 57 (1.7) | 8 (1.3) | 0 (0) | 3 (9.1) | 24 (4.5) | 1 (0.5) | 15 (2.5) | 6 (1) | |
| Species, n (%) | | | | | | | | | |
| *Pf* | 35 (61.4) | 5 (62.5) | – | – | 17 (70.8) | 1 (100) | 8 (53.3) | 4 (66.7) | p = 0.002 |
| *Pv* | 13 (22.8) | 2 (25) | – | – | 7 (29.2) | – | – | 1 (16.7) | |
| *Pm* | 5 (8.8) | – | – | – | 4 (26.7) | 1 (16.7) | |
| *Pf* & *Pv* | 1 (1.8) | 1 (12.5) | – | – | – | – | – | |
| *Pf* & *Pm* | 3 (5.3) | – | – | – | – | – | 3 (20) | – | |

*P. falciparum* – *Pf*; *P. vivax* – *Pv*; *P. malariae* – *Pm*; *P. falciparum* and *P. vivax*—*Pf* & *Pv*; *P. falciparum* and *P. malariae*—*Pf* & *Pm*. * Fisher’s exact test
detected in the participants’ blood samples. Out of the 57 PCR positive cases, three presented with clinical malaria within a period of two years after recruitment, including one case of acute vivax malaria seven months after recruitment (personal communication, NVT).

By occupation, PCR positive samples were detected in 2.0% (35/1737) of farmers, 3.1% (10/323) of students and 1.0% (12/1223) of government workers (Additional file 1: Table S2, Fisher’s exact  \( p = 0.156 \)). No significant differences in PCR malaria prevalence were seen by age group (Additional file 1: Table S3, Fisher’s exact  \( p = 0.523 \)), sex (Additional file 1: Table S4, Fisher’s exact  \( p = 0.312 \)) or ethnicity (Additional file 1: Table S5, Fisher’s exact  \( p = 0.803 \)).

**Molecular markers of drug resistance for *P. falciparum* malaria**

Molecular markers of drug resistance were analysed in samples with sufficient amount of DNA for PCR amplification and sequencing. No samples had mutations associated with artemisinin resistance in *Pfkcalch*-13 (0/13). Among markers associated with piperaquine resistance, none of the samples had the *exo-E415G* mutation (0/11), but 13.3% (2/15) had increased copy numbers of plasmepsin 2/3. Chloroquine resistant haplotype CVIET at positions 72–76 of PfCRT gene was observed in 27.3% (6/22) samples. Four out of 15 samples (26.7%) had multiple copies of *Pfdmrd1*. The two isolates with multiple plasmepsin 2/3 copies also had multiple copies of *Pfdmrd1* gene (2/15, 13.3%).

**Serological findings**

Overall, 38.5% (1257/3262) of participants had antibodies to at least one *P. falciparum* antigen, and 31.1% (1022/3282) responded to at least one *P. vivax* antigen (Fig. 2 and Additional file 1: Table S6). The highest seroprevalence for individual antigens of each species was 30.6% (1001/3274) for PfAMA1 and 18.7% (614/3283) for PvAMA1. In contrast, the lowest seroprevalence was found against PfMSP1 (14.4%, 471/3277) and PvMSP1 (11.1%, 365/3283), respectively. Seroprevalence varied significantly by district (Additional file 1: Table S6; Pearson’s Chi-Square test  \( p < 0.001 \)) and were highest in Krong Chro’s Chi-Square test significantly by district (Additional file 1: Table S6; Pearson’s Chi-Square test  \( p < 0.001 \)). Seroprevalence varied against PfMSP1 (14.4%, 471/3277) and PvMSP1 (11.1%, 365/3283), respectively. Seroprevalence varied significantly by district (Additional file 1: Table S6; Pearson’s Chi-Square test  \( p < 0.001 \)) and were highest in Krong Chro’s Chi-Square test significantly by district (Additional file 1: Table S6; Pearson’s Chi-Square test  \( p < 0.001 \)).

Factors associated with *P. falciparum* and *P. vivax* exposure (defined as being seropositive to any antigen for each species) were determined using logistic regression in five of the study districts (Table 4). In these areas, 52% of the participants had evidence of previous exposure to *P. falciparum* (1178/2279) and 42% (962/2282) to *P. vivax*. There was no association between PCR positivity (*P. falciparum*: OR 0.88 [95% CI 0.50–1.55],  \( p = 0.660 \); *P. vivax*: OR 0.72 [95% CI 0.40–1.31],  \( p = 0.287 \)) and antibody responses. Female sex, being from an ethnic minority group, farming or residing in Krong Pa and Kong Chro districts indicated an increased risk of both *P. falciparum* and *P. vivax* exposure ( \( p < 0.001 \)). Overall, there was an increased risk of seropositivity for older age groups as compared to young children, especially for *P. falciparum*.

Seroprevalence data were used to estimate malaria transmission intensity by fitting age-seroprevalence curves for *P. falciparum* and *P. vivax* exposure at each district (Figs. 3 and 4), as well as for response to individual antigens (Additional file 1: Figure S2 for *P. falciparum* antigens and Additional file 1: Figure S3 for *P. vivax* antigens). Seroconversion rates were highest in Krong Pa and Kong Chro ( \( \lambda \) range for *P. falciparum* = 0.063–0.162; \( \lambda \) range for *P. vivax* = 0.066–0.223), the districts with the highest prevalence of clinical malaria in 2014–2015 (Figs. 3 and 4, Additional file 1: Table S1 and Additional file 1: Table S7). In contrast, seroconversion was lower in Chu Prong, Duc Co and la Grai districts ( \( \lambda \) range for *P. falciparum* = 0.009–0.031; \( \lambda \) range for *P. vivax* = 0.004–0.008; Additional file 1: Table S7). In these three districts, responses elicited against PfAMA1 and PvAMA1 were the main contributors to overall exposure data, as the number of individuals seropositive for MSP1 and/or CSP antigens was low (Additional file 1: Figure S2 and Additional file 1: Figure S3).

**Discussion**

Sub-clinical malaria represents a disease reservoir that should be considered when determining the malaria risk for naïve people, and for developing malaria control and elimination strategies in areas transitioning from pre-elimination to elimination. This is the first study to provide a snapshot of the burden of asymptomatic malaria in Gia Lai province, Vietnam.

The overall prevalence of asymptomatic malaria in the 3283 participants was 1.74% in December 2016–January 2017, with 96.4% (55/57) below the RDT detection threshold. This prevalence was markedly higher than the mean clinical malaria prevalence rate of 0.12% (108/90,395) reported in 2016 among residents from the 14 communes where the participants were recruited. Although the survey included a subset of the province population, other studies in similar epidemiological
settings have indicated that asymptomatic *Plasmodium* carriers are substantially more prevalent than clinical cases [8, 9]. Moreover, the district selection covered geographically distinct areas, as well as people from all age groups, minority ethnicities, and different occupations. In the case of Chu Puh and Chu Se districts the recruitment included mainly adults from military forces, which could suggest a bias in the asymptomatic malaria prevalence estimates. However, both districts are semi-urban areas where a decrease in overall malaria cases has been observed in the past years and thus a lower prevalence of asymptomatic infections could be expected.

Importantly, the PCR assay in the present study using a finger prick capillary blood of approximately 10 µL and based on the PCR performance acceptance threshold was designed to detect sub-microscopic malaria infections with a sensitivity of at least 1.6 to eightfold greater than that of microscopy (the LOD in thick blood films for

![Fig. 2 Seroprevalence of participants in seven districts in Gia Lai province against *P. falciparum* and *P. vivax* antigens. Error bars indicate 95% CI](image-url)
expert microscopists is estimated at 4 to 20 parasites/µL [30]). Moreover, we cannot exclude that the sub-microscopic reservoir was larger if using alternate molecular methods that offered greater sensitivity, such as ultra-sensitive quantitative PCR assays that use high blood volume (2–3 mL of venous blood; LOD as low as 22 parasites/mL [9, 31], or assays targeting multi-copy gene families (LOD 30–150 parasites/mL) [32]. In this study, finger-prick capillary blood collection followed by standard PCR was selected to minimize the burden on study participants (reluctance to provide venous blood) and reduce logistic requirements and costs, both of which would have impacted on the conduct of present study as well as potential feasibilities of future surveys.

The results suggest no presence of *P. ovale* and *P. knowlesi* infections in the study population. In the present study, the PCR assay sensitivity was only tested for *P. falciparum*. Although rare, *P. ovale* infection detected by PCR has been reported in several southern Vietnam provinces (Song Be, Lam Dong, Dak Lak, and Khanh Hoa) [33] and in south-central Ninh Thuan province [34], but the species has not been reported in Gia Lai yet. Similarly, *P. knowlesi* has been reported in south-central Ninh Thuan province [35] and in central Quang Tri province [36], but not in Gia Lai. The detection of cases of asymptomatic *P. malariae* (three mixed infections of *P. falciparum* and *P. malariae*, and five mono-infections of *P. malariae*) supports other reports of cryptic or asymptomatic *P. malariae* infections in Southeast Asia [37]. The clinical and public health importance of these asymptomatic infections is unknown, but the prevalence may be sufficient to maintain transmission in the study area and warrants further investigation.

Table 4 Factors associated with *P. falciparum* and *P. vivax* seropositivity to any antigen in the study area, Gia Lai province (excluding Chu Puh and Chu Se districts)

| Variable        | Any *P. falciparum* antigen | Any *P. vivax* antigen |
|-----------------|-----------------------------|-----------------------|
|                 | Seropositivity OR (95% CI)  | Seropositivity OR (95% CI)  |
|                 | n/N Rate (%)                | n/N Rate (%)          |
| PCR             |                             |                       |
| Negative        | 1163/2230 52.1              | 945/2233 42.3         |
| Positive        | 24/49 48.9 0.88 (0.5–1.55)  | 17/49 34.7 0.72 (0.40–1.31) |
| Age group       |                             |                       |
| 5–9y            | 90/223 40.4 0.88 (0.5–1.55) | 77/224 34.4           |
| 10–19y          | 11/20 55.0 1.81 (0.72–4.54) | 12/20 60.0 2.86 (1.12–7.3) |
| 20–29y          | 31/116 26.7 0.54 (0.33–0.88) | 26/116 22.4 0.55 (0.33–0.92) |
| 30–39y          | 46/75 61.3 2.34 (1.37–4.01) | 38/74 51.3 2.01 (1.18–3.43) |
| 40–49y          | 38/62 61.3 2.34 (1.31–4.17) | 29/62 46.8 1.68 (0.94–2.97) |
| 50–59y          | 17/34 50.0 1.48 (0.72–3.04) | 15/34 44.1 1.51 (0.73–3.13) |
| 60–69y          | 109/176 61.9 2.4 (1.6–3.61) | <0.001 88/177 49.7 1.89 (1.26–2.83) |
| Sex             |                             |                       |
| Male            | 529/1164 45.4 1.73 (1.46–2.04) | 436/1166 37.4         |
| Female          | 658/1115 59.0 1.73 (1.46–2.04) | 526/1116 47.1 1.49 (1.26–1.73) |
| Ethnicity       |                             |                       |
| Kinh            | 91/408 22.3 1.23 (0.86–1.78) | 67/408 16.4           |
| Minority        | 1096/1871 58.6 4.93 (3.83–6.33) | 895/1874 47.8 4.65 (3.53–6.14) |
| District        |                             |                       |
| Chu Prong       | 97/327 29.7 1.48 (0.72–3.04) | 59/329 17.9           |
| Duc Co          | 147/530 27.7 0.91 (0.67–1.23) | 67/530 12.6 0.66 (0.45–0.97) |
| Ia Grai         | 75/219 34.2 1.23 (0.86–1.78) | 58/220 26.4 1.64 (1.09–2.47) |
| Krong Pa        | 370/603 61.4 3.76 (2.82–5.02) | 328/603 54.4 5.46 (3.95–7.54) |
| Kong Chro       | 498/600 83.0 11.58 (8.42–15.92) | 450/600 75.0 13.73 (9.8–19.22) |
| Occupation      |                             |                       |
| Farmer          | 1015/1734 58.5 0.56 (0.44–0.71) | 798/1736 46.0         |
| School age      | 142/322 44.1 0.56 (0.44–0.71) | 138/323 42.7 0.88 (0.69–1.11) |
| Worker/Soldier  | 30/223 13.5 0.11 (0.07–0.16) | 26/223 11.7 0.16 (0.1–0.24) |

*p Univariate logistic regression*
Among asymptomatic infections caused by *P. falciparum* no *Pfkelch*-13 mutations that confer artemisinin resistance were detected (0/13). This finding contrasts with other recent studies that reported a prevalence of *Pfkelch*-13 validated mutations of 74% (20/27) in Ia Grai district in 2015, and up to 89% (39/45) in Krong Pa district in 2017, in both cases C580Y mutations were predominant [19, 20]. Unlike the present study, both studies by Thanh et al. and Rovira-Vallbona et al. were clinical trials conducted with symptomatic individuals presenting with high parasite densities (> 500 parasites/μL) [19, 20]. The absence of *Pfkelch*-13 mutations in samples collected from asymptomatic individuals in the present study could be due to the small
sample size of the subset of *P. falciparum* positive samples (13/35) for which the sequence data were obtained and/or heterogeneity in the local parasite populations. Another possible explanation is that in the absence of drug pressure resistant parasites appear to be less fit in comparison with the wild type parasites [38], and thus could be outgrown by the wild type parasites in low parasitemia asymptomatic infections. On the other hand, multiple copies of the *plasmepsin 2/3* genes and *pfmdr1* gene were found in 13.3%, (2/15) and 26.7% (4/15) of infections, respectively, including two double mutants. Amplification of *plasmepsin 2/3* confer resistance to piperaquine, the partner drug in the first-line artemisinin-combination therapy DHA-PPQ used

Fig. 4 Age-seroprevalence curves for *P. vivax* exposure across five districts in Gia Lai province, Vietnam. Exposure is defined as seropositivity to at least one *P. vivax* antigen. Reversible catalytic conversion models allowing one seroconversion rate (λ) were fit to the data (dashed line indicates 95% CI)
in Vietnam up until 2020. The rate of plasmepsin 2/3 amplification coincides with that reported in a clinical trial conducted Krong Pa district during the same period in Vietnamese subjects (10.4%, 5/48) [20]. Increases in the pfmdr1 copy number are associated with resistance to mefloquine, which has not been used in the study area since the 1990s (personal communication with Dr. Ngo Duc Thang). This suggests the possibility of importation of resistant malaria parasite from neighboring Cambodia, where artesunate-mefloquine has been used to treat *P. falciparum* malaria in early 2000s [39]. Further investigation is needed to better understand the movement of individuals carrying malaria parasites across the international border that Gia Lai province shares with Cambodia.

This study also aimed to explore serology as an alternative malaria transmission metric for surveillance purposes. The antigens AMA-1, MSP1 and CSP, were selected as they are present in both species, represent both pre-erythrocytic and erythrocytic stage parasites and have been extensively used as markers of exposure in the past. The analysis of antibody responses of study participants found that 38.5% (1257/3262) and 31.1% (1022/3282) of individuals responded to at least one *P. falciparum* or *P. vivax* antigen, respectively. Although no other serological surveys had been conducted previously in Gia Lai, these rates compare to those found in similar epidemiological settings, such as a population survey in Quang Nam, Vietnam, in 2015 (42.3%, 145/343 for *P. falciparum* and 22.4%, 77/343 for *P. vivax*), or a study among malaria-negative adults in Oddar Meanchey, Cambodia, in 2011 (50%, 57/113 for *P. falciparum-*MSP1 and 61%, 69/113 for *P. vivax*-MSP1) [18, 40]. The relatively high seroprevalence rates as compared to parasitological estimates could be explained by (1) the fact that antibody responses reflect cumulative exposure events in the past, including asymptomatic infections, (2) the relatively long half-life (i.e. >6 months) of responses against Pf/PvAMA1 or Pf/PvMSP1 antigens generated after exposure events [41, 42] and, to a minor extent, (3) missed sub-microscopic infections in the cross-sectional survey that could increase population malaria prevalence rate. Antibody decay rates (or half-lives) for the antigens used in the present study had been previously determined in a cohort from Ratanakiri province, Cambodia (bordering Gia Lai province) using multiple antibody measurements over 2 years’ time; the authors reported half-lives to be between ≈ 10 and 28 months in individuals that remained PCR negative during the follow-up (i.e. 297 days for PfMSP1, 569 for PfCSP, 367 for PvAMA1, 312 for PvMSP1, 885 for PvCSP210 and 725 for PvCSP247) [42]. The long half-live against CSP, particularly for the two *P. vivax* antigens, may partly explain the high seroprevalence found for CSP antigens in some of the districts included in the present study.

Age-seroprevalence curves allowed to identify two types of districts. For Chu Prong, Duc Co, and La Grai districts a linear association between age and seroprevalence rate was seen, suggestive of a sustained decrease in malaria prevalence in the past years. In contrast, Krong Pa and Kong Chro districts had the highest seroconversion rates for all antigens and a rapid acquisition of antibodies at early ages for both *P. falciparum* and *P. vivax* antigens, what can be interpreted as ongoing transmission of both species. Although the higher number of individuals <20 years of age in Krong Pa and Kong Chro might have contributed to these differences, retrospective data showed these two districts had the highest clinical malaria rate among study districts in the two years preceding the survey, in line with serological results. The decrease in malaria cases reported between 2014 and 2016 in all districts by an average of 6.4-fold was not captured in the age-seroprevalence curves conducted at the end of 2016, probably due to the high immunogenicity and long duration of responses to selected antigens.

Risk factors for seropositivity were assessed after excluding the two districts with only military personnel and negligible seropositivity rates (i.e. Chu Puh and Chu Se). Even so, district of residence significantly increased risk of seropositivity for both *Plasmodium* species, in agreement with the higher number of malaria cases that detected in Krong Pa and Kong Chro communal health stations in recent years (Tables 2 and 4). *Plasmodium falciparum* antibody prevalence was also highest among older age groups, what may be explained by different risk behaviour compared to younger student-age groups (i.e. working in farming and sleeping outdoors) together with their history of exposure to infection during their lifetime. Similarly, the higher seroprevalence in females than males may be due to a higher proportion of females being farmers by occupation (83.8%; 936/1117) compared with males (68.7%; 801/1166) in these communities. Farming activities often include going far into forests where risk of malaria is higher. On the other hand, no association was seen between PCR positivity in the cross-sectional survey and *P. falciparum* or *P. vivax* seroprevalence. Although studies in low endemic areas have shown that even low-density asymptomatic infections can induce strong long-lived IgG responses against *P. vivax* [43], the number of PCR positive individuals was low. Another study using qPCR for parasite diagnosis in Quang Nam province could not identify active parasite carriers despite serological evidence of ongoing transmission in consecutive cross-sectionals [18]. As compared to parasite prevalence surveys that can only identify active infections, serological surveys in pre-elimination settings
can be cost-effective tools to confirm the cease in transmission in a given area, and/or to measure changes in transmission intensity over time.

Conclusions
Sub-clinical malaria represents a disease reservoir that should be considered when planning malaria elimination strategies and for determining the malaria risk for naïve personnel, such as travelers and military personnel visiting malaria endemic areas. In this study, the asymptomatic prevalence of malaria infections was 1.74% in seven districts of Gia Lai province, an area of low clinical malaria endemicity. The high seropositivity rates for *Plasmodium* antigens suggest that a significant proportion of participants had previous malaria exposure, and age-dependent trends allowed to differentiate districts with sustained low transmission from others with higher ongoing transmission levels of both *P. falciparum* and *P. vivax*. The results of this study can inform NMCP on the burden of asymptomatic malaria in Gia Lai and provide tools for geographical stratification of transmission, in order to design efficient public health and elimination strategies.

Abbreviations
- CI: Confidence interval
- DHA-PPQ: Dihydroartemisinin-piperaquine
- ELISA: Enzyme-linked immunosorbent assay
- GMS: Greater Mekong Subregion
- ITN: Insecticide-treated net
- LQD: Limit of detection
- NMPE: National Institute of Malariology, Parasitology and Entomology
- NMCP: National Malaria Control Programme
- OD: Optical density
- OR: Odds ratio
- PBS: Phosphate buffered saline
- PCR: Polymerase chain reaction
- PQR: Prevalence odds ratios
- qPCR: Quantitative real-time PCR
- RDT: Rapid diagnostic test
- WHO: World Health Organization

Supplementary Information
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Authors’ contributions
NDM and NNS made equal contribution to the study. NJM, NNS, NDM, and MDE designed the study and developed the protocol. NNX obtained approval of military health facilities to support the cross-sectional survey. NNS and NJM obtained scientific and ethical approval for the study. NNS, NVT and TKL carried out the field work. MC provided direction, training, and quality assurance of the PCR assays. NHB performed the serological assays with scientific contribution from KAE. ERV and NH8 analysed the serological data and ERV critically reviewed the serological findings. NJM, ERV, MC and MDE wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
Ethics review was conducted by the Vietnam National Institute of Malariology, Parasitology and Entomology Institutional Review Board (2016-VM-P5) and extra-mural review was conducted in accordance with U.S. Department of the Navy Human Research Protection Program guidelines (HRPO.NMRCA.2017.0003) in compliance with all applicable federal regulations governing the protection of human subjects. Written informed consent was obtained from participants and parents or guardians of minor participants prior to enrolment. Children aged 10 to 17 years of age provided assent to participate in the study.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interest.

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