Asymptomatic Malaria Infections in an Endemic City of Honduras

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ABSTRACT

BACKGROUND: This study aimed at determining the prevalence of asymptomatic cases of malaria in Puerto Lempira, the municipality with the highest morbidity in Honduras.

METHODS AND FINDINGS: Capillary blood was collected from 1,899 participants. All samples were analyzed through microscopy and a polymerase chain reaction (PCR) technique based on the amplification of species-specific repetitive sequences. The molecular approach was more sensitive than microscopy to detect asymptomatic infections (1.1% vs. 0.16%).

CONCLUSIONS: Although the prevalence of asymptomatic malaria was low in this population, new strategies such as active case detection and PCR-based surveys could become useful tools for countries aspiring to initiate the pre-elimination phase of malaria in the Americas.

KEYWORDS: asymptomatic malaria, Honduras, Mesoamerica

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Introduction

The number of malaria cases in Honduras between 2004 and 2014 has decreased from 17,000 to 3,380, a fivefold decline (Fig. 1).[1] Similar trends are observed in most Latin American countries, many of which have already surpassed the targets set by the Millennium Development Goals established for 2015. Due to these achievements, a recent Regional initiative including Mesoamerica and the Island of Hispaniola is supporting efforts for the elimination of malaria by 2020.[2] Of the 10 countries comprising this initiative, three are in the pre-elimination phase while Honduras and six other countries are in the control phase, but aspiring to move toward the elimination phase. However, for some countries, this involves significant improvements in malaria epidemiological surveillance. New strategies such as active case detection (ACD) as well as surveys using molecular diagnostic tools are urgently needed.

Compared to microscopy, polymerase chain reaction (PCR)-based techniques show superior sensitivity in detecting infections below the threshold permitted by thick smear,[3–5] either because patients are in the prodromal period or because they are asymptomatic carriers harboring low-density infections. Also, PCR-based diagnosis is useful for monitoring the decline of the prevalence over time, especially in areas aiming at malaria elimination.[6]

Despite significant progress in reducing malaria transmission in most of the country's 18 departments, a high incidence of malaria remains in those located along the Honduran Caribbean coast. Among the latter, two departments, Colón and Gracias a Dios, contribute with 74% of the malaria cases and concentrate almost all cases of *Plasmodium falciparum*. Located in Gracias a Dios, Puerto Lempira, is a municipality with an area extension of 8,063 km^2 and a population of over 25,000 people (Fig. 2). This municipality continues to be a high transmission territory, with an average of 50 cases per 1,000 inhabitants (more than 10 cases per 1,000 inhabitants is classified as high transmission area, while 1–10 cases and less than 1 case/1,000 inhabitants are classified as moderate and low, respectively). Moreover, the number of cases between 2012 and 2013 increased from 870 to 1,705, respectively (Fig. 1). The Ministry of Health of Honduras has responded to this challenge by implementing a comprehensive intervention and monitoring plan throughout 2014. Such activities involved community education, household mapping, distribution of long-lasting insecticide-treated nets, and an ACD survey to detect asymptomatic

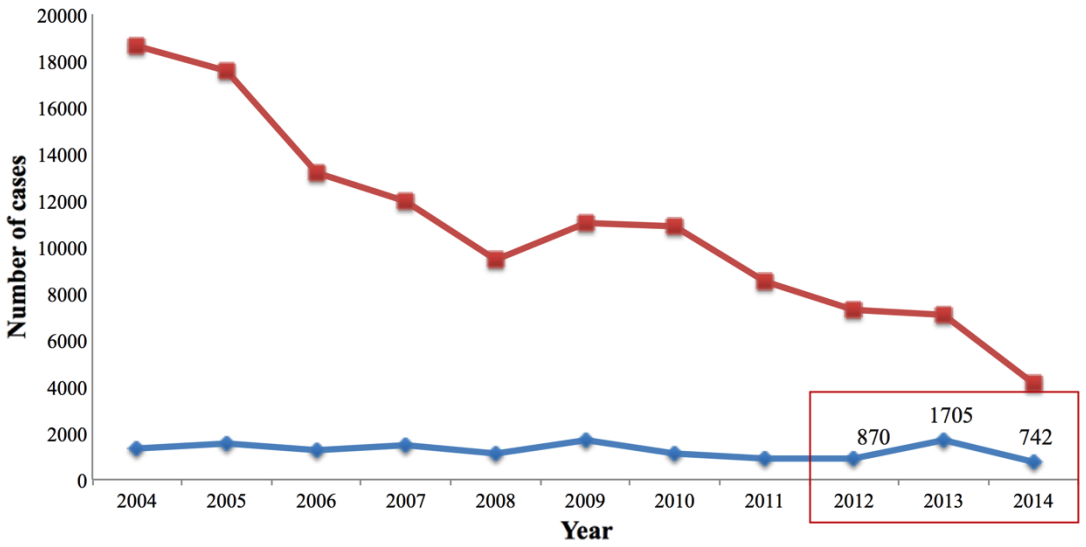

Figure 1. Malaria prevalence from Honduras and Puerto Lempira.
Note: Honduran Ministry of Health and World Malaria Report 2014.[1]

individuals in a subsample of the population residing in the most at-risk neighborhoods. This report presents the findings of the ACD survey.

Methods

A total of 1,899 persons [722 (38%) females] participated in the ACD survey, which included noncare-seeking people of any age and sex who lived in 13 neighborhoods of Puerto Lempira city (Fig. 2) and without malaria symptoms. For the selection process of participants, a household-based survey was conducted using convenience sampling. After informed consent of participants, blood samples were obtained by finger pricks, and blood drops were used to make one thick smear to be analyzed by microscopy and one blood spot on Whatman filter paper for molecular analysis. Samples were collected during the rainy season in August 2014, when transmission rates were declining. Experienced and certified microscopists performed the diagnosis of malaria according to national guidelines[7] and protocols recommended by the Pan American World Organization. These microscopic analyses were carried out in the Regional Reference Malaria Laboratory of Honduras. A second microscopist confirmed the results for the entire number of thick smears. Molecular diagnoses were performed extracting DNA from a 3 mm disk containing dried blood by a Chelex-100 based method (Bio-Rad Laboratories) and using a single-tube species-specific PCR targeting genomic

Figure 2. Honduras map showing the location of Puerto Lempira city.

consensus repeat sequences of *Plasmodium* sp. named Pvr47 and Pfr364.[8]

Briefly, amplifications were performed under the following conditions in a 25 µL volume: 12.5 µL of a 2 × master mix (Promega Corporation), 10 pmol of the primers Pf 7142: 5′-GCTTTGAAGTGCATGTGAATTGTGCAC-3′ and Pf 7178: 5′-CCGGAAATTCGGGTTTTAGAC-3′ for *P. falciparum*; and 12.5 pmol of the primers Pv 7074: 5′-CAAATGTAGCATAAAAATCYAAG-3′ and Pv 7175: 5′-CTGATTTTCCGCGTAACAATG-3′ for *Plasmodium vivax*. A total of 40 ng of DNA were used in all PCR reactions. Reactions were amplified by an initial denaturation at 95°C for 2 minutes, 35 cycles of 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 5 minutes. Amplicons were visualized by 2% agarose gel electrophoresis with ethidium bromide. Expected size products were 220 bp for *P. falciparum* and 333 bp for *P. vivax*.

A total of 10% of PCR-negative samples and all PCR-positive samples were confirmed by running the PCR again and by a second PCR protocol targeting the 18S ribosomal gene of the parasite, which is a widely accepted approach because of its sensitivity.[9] This nested PCR was performed in 50 µL as total volume containing 25 µL of Promega 2 × master mix, 20 pmol of rPLU1 and rPLU5 primers (5′-TCAAAGAT TAAGCCATGCAAGTGA-3′/rPLU5: 5′-CCTGTTGTT GCCTTAAACTYC-3′) and 40 ng of DNA. The first PCR was followed by a species-specific PCR amplification. Reaction mixtures were composed of 25 µL of 2 × master mix and 20 pmol of each primer (rFAL1/rFAL2 for *P. falciparum* and rVIV1/rVIV2 for *P. vivax*), until the final volume was 50 µL. Cycling conditions were as follows: 1 cycle at 94°C for 4 minutes; 35 cycles at 94°C for 30 seconds, 58°C for 1 minute, and 72°C for 1 minute; and a final extension step was carried out at 72°C for 4 minutes. The observed amplicon sizes were 205 bp for *P. falciparum* and 107 bp for *P. vivax*. Products from the second PCR were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. The sequences of the primers are: rFAL1: 5′-TTAAACTG GTTTGGGAAAACCAAATATATT-3′; rFAL2: 5′-ACA CAATGAACTCAATCATGACTACCCGTC-3′; rVIV1: 5′-CGCTTCTAGCTTAATCCACATAACTGATAC-3′; and rVIV2: 5′-ACTTCCAAGCCGAAGCAAAGAAAG TCCTTA-3. Negative and positive controls were run in each reaction.

The presence of parasitemia was confirmed when an expected band size corresponding to *P. falciparum* and *P. vivax* were present.

Diagnostic performance was evaluated for each test calculating the sensitivity, specificity, and predictive values with either test as the reference standard. The agreement between the results of both techniques was assessed using the Cohen's kappa (κ) index and its 95% CI.[10] The proportions were compared using a two-tailed Fisher's exact test.

These results are product of an intervention carried out by the Ministry of Health and Panamerican Health Organization (PAHO) as part of the routinary national surveillance strategies. Therefore, they are not considered as a research study. This ACD survey was approved by the national and local health authorities and obtained the agreement of all the participants and of their legal guardians. It also followed all ethical standards established by the National Ethics Committees. The research was conducted in accordance with the principles of the Declaration of Helsinki.

Results and Discussion

The microscopic analysis detected three positive samples, for a prevalence of 0.16%. Two cases were due to *P. vivax* while the third one was due to *P. falciparum* (Table 1). Parasite densities for both *P. vivax* infections were low and ranged from 132 to 348 parasites/µL, while the *P. falciparum* infection was classified as moderate, with 1,200 parasites/µL. On the other hand, the molecular approach detected 21 infections (including all three positive by microscopy), for a prevalence of 1.1%. Of those, 14 cases were due to *P. vivax* while the remaining seven cases were due to *P. falciparum*. Within the positive samples, both microscopy and PCR produced a 2:1 ratio between *P. vivax* and *P. falciparum*. This is a reasonable and expected ratio based on accumulated case reporting in the study area (276 *P. falciparum* cases versus 903 total malaria cases by September 2014). The age of the asymptomatic carriers ranged from 1 to 73 years, with an average of 27.3 years [9 (43%) individuals were younger than 18 years old]. Sixteen (76%) of 21 positive individuals were females, whereas five (24%) were males. These results reveal that the prevalence of asymptomatic carriers of malaria was low at the time of the study in this population.

In Honduras, a previous study was conducted in 1998 among 319 individuals in Tocoa (Colón), a city with epidemiological characteristics similar to that of Puerto Lempira. That study showed that 16.61% of asymptomatic infections were estimated by rapid diagnostic tests and 14.73% through microscopy.[11] In 2002, a second study in Gracias a Dios among 146 school children showed a prevalence of 1.4% of asymptomatic *P. vivax* carriers detected only through microscopy.[12]

Table 1. Asymptomatic malaria cases diagnosed by thick drop microscopy and polymerase chain reaction.

	P. vivax	*P. falciparum*	MIXED	NEGATIVE	PREVALENCE	SENSITIVITY	SPECIFICITY	PPV	NPV
Microscopy	2	1	0	1896	0.16%	14.3%	100%	100%	99.1%
PCR	14	7	0	1878	1.11%	100%	99.1%	14.3%	100%

Note: Sensitivity, specificity, and predictive values are shown using either test as the reference standard.

More recently, a cross-sectional study was conducted among 2,554 school going children from all over the country, and only 0.2% of them revealed an asymptomatic malaria vivax infection in Gracias a Dios.[13] Although this trend may reflect a real decline in asymptomatic infections associated with a lower prevalence of malaria in the country over the last two decades, it may also be due to a different epidemiology and a different risk of transmission for scholars and adults.

A 100% of concordance was established among the screening and confirmatory PCR methods, for 10% of the negative and all positive samples. As listed in Table 1, performance data were calculated with either test as the gold standard. Microscopy showed a very low sensitivity (14.3%) as compared to the PCR test, which is lower than expected for the traditional gold standard technique in similar settings.[14] A kappa value of 0.248 (CI = 0.218–0.278) confirms a poor concordance between microscopy and PCR results. The two-tailed Fisher's exact test revealed a *P*-value = 0.0003, which is interpreted as extremely statistically significant and supports the fact that PCR is able to detect a higher number of asymptomatic infections than microscopy under the current circumstances. Those results are in agreement with several authors reporting that PCR-based molecular techniques are more efficient than microscopy for asymptomatic malaria infections.[15–17]

Several experimental studies reveal different prevalence rates of asymptomatic infections among geographic regions with malaria transmission, which varies from 0% in Sri Lanka and Iran[18,19] to 39% in some African countries,[20–22] or as high as 70% in a population of miners from Mato Grosso in Brazil.[23] Also, other authors reported prevalence rates similar to those found in our study in populations of Asia and Africa.[3,24] Due to this high heterogeneity, it seems necessary to investigate the role of asymptomatic malaria infections in the maintenance of the parasite in each endemic area of the world. In consequence, the best strategies for its detection should be defined based on local results, because of their potential role as reservoirs[14,19,25] and the threat they might represent to the success of the surveillance programmes when a country is approaching to the elimination phase.[26,27] For settings of low endemicity, some meta analysis reports provide robust information concerning the increasing importance of submicroscopic parasite carriage as the malaria transmission intensity is reduced.[14,17,28]

Although malaria control efforts have led to substantial reduction in the number of cases in Honduras, low-level transmission continues to occur. Malaria diagnosis in Honduras is mainly based on thick drop smears, although RDTs are used on a smaller scale at locations lacking an appropriate facility. In addition, the National Malaria Surveillance Program has implemented confirmatory PCR techniques as an external quality control to assess the microscopy once a year (since 2012). Although these molecular results are useful to improve microscopic diagnosis, they have two disadvantages: they do not provide opportune information for patients with a false-negative result by thick drop and do not detect asymptomatic infections since only febrile patients are evaluated.

Although it seems clear that PCR-based techniques have a higher sensitivity than microscopy,[29,30] the current approach do not seem to contribute directly to the interruption of malaria transmission in Honduras due to its inability to detect asymptomatic infections. Therefore, the implementation of routine and frequent ACD PCR-based surveys could be a good strategy for Honduras, and also for Mesoamerica and the island of Hispaniola. However, no matter the strategies chosen by the elimination programmes, it would be important to avoid a relaxation in control efforts once the prevalence diminishes by conventional microscopy.[14]

Conclusions

Although the prevalence of asymptomatic infections is low in this population, these carriers could contribute to keep the transmission of malaria and hinder the progress of the national and regional surveillance programmes. Due to the low sensitivity of the thick smear, it is suitable to implement techniques based on PCR for the detection of asymptomatic infections.

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Author Contributions

Conceived and coordinated the study: GAF and REM-T. Carried out the molecular techniques: AM and JSG. Coordinated and carried out the microscopic diagnosis: LAEP and JM. Participated in the design of the study and coordination and helped to draft the manuscript: LE. All the authors read and approved the final manuscript.

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