

Short Communication

Absence of asymptomatic cases of malaria in a historically endemic indigenous locality of the Department of Caaguazú, Paraguay: moving toward elimination

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Abstract

Introduction: Paraguay was among the 16 countries that reported zero indigenous malaria cases in 2014. **Methods:** A cross-sectional observational descriptive study was performed in 100 adults from Santa Teresa, Paraguay. Parasite detection was carried out using seminested multiplex polymerase chain reaction (PCR) and microscopy. **Results:** Among the participants, 44% were female and 56% were male, and 89% had a malaria history. No parasites were detected with either of the methods. **Conclusions:** There were no asymptomatic cases in Santa Teresa, and this finding is very promising. A longitudinal study should be performed to confirm that there are no asymptomatic cases in this locality.

Keywords: Asymptomatic cases. Malaria. Paraguay.

Malaria is a disease transmitted to humans through a bite from a female mosquito of the genus *Anopheles* infected with parasites of the genus *Plasmodium*, such as *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium vivax*. It is estimated that, in 2010, there were 216 million cases of malaria in the world and that over 150,000 deaths were caused by this disease⁽¹⁾. In 2014, Paraguay was among the 16 countries that reported zero indigenous cases⁽²⁾.

According to records, in the 1940s, 90% of Paraguay frequently experienced cases of malaria, and 82% of the cases were noted in the following three departments in the eastern region of the country: Alto Paraguay, Caaguazú, and Canindeyú. Owing to large efforts in controlling the disease, the number of cases decreased sharply from 6,853 cases in 2000 to 1,392 cases in 2003 (79% reduction)⁽³⁾. Since 2003, the control of malaria has been considered successful, with only 91 cases in 2009 and 23 cases between January and July 2010 (16 of these were autochthonous cases). Among the cases, 94% were from the department of Alto Parana and 6% were from the department of Caaguazú⁽³⁾.

In Paraguay, the last case of autochthonous transmission of malaria was reported in 2011, and Paraguay was honored with the title of *Malaria Champions of the Americas 2012* by the Pan American Health Organization (PAHO). This title placed

the country among the six countries in the pre-elimination phase of the disease in Latin America⁽⁴⁾.

Currently, the National Service for elimination of the disease (SENEPA) considers the Alto Paraná and Caaguazú departments, which were historically endemic regions, as regions with low risk of transmission⁽³⁾.

In the locality of Santa Teresa in the district of Raul Arsenio Oviedo of the department of Caaguazú, 134 cases of infection with *P. vivax* (30% of its population) were reported in 2007, just before the epidemiological silence started (unpublished data of the SENEPA). On the other hand, the World Health Organization (WHO) reported the existence of a high prevalence of asymptomatic cases with *P. vivax*, and the operational difficulties concerning the treatment of hypnozoites (latent stage of *P. vivax*) indicate that the probability of *P. vivax* elimination is lower than that of *P. falciparum* elimination⁽⁵⁾. The elimination of hypnozoites is clearly the main challenge in malaria elimination programs, and although there are preclinical studies on *P. vivax* vaccines currently, this stage of the parasite is an important factor for the control of the disease.

The focus of malaria management is moving toward low-density, subclinical infections and geographically and demographically concentrated reservoirs. Molecular diagnoses have shown that this asymptomatic parasitic reservoir is more widespread than previously thought, even in low-endemic areas. When transmission reaches very low levels, submicroscopic carriers are estimated to be the source of 20-50% of all human-to-mosquito transmissions⁽⁶⁾.

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Plasmodium parasites can be identified by microscopy, which is the gold standard method. Additionally, they can be identified using seminested multiplex-polymerase chain reaction (SnM-PCR), which is a molecular diagnostic approach that has been shown to be useful for the detection of subpatent parasitemias. SnM-PCR can detect the following four species of *Plasmodium* that infect humans and cause malaria: *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. SnM-PCR amplifies the 18S small subunit ribosomal deoxyribonucleic acid (ssrDNA) of the parasite, which is present in multiple copies, with conserved and non-conserved regions that are characteristic of the four species⁽⁷⁾. This molecular method has been previously very useful, because it allowed the elucidation of an imported case of malaria from West Africa to Paraguay⁽⁸⁾. This case was initially mistakenly diagnosed using microscopy as an infection of *P. vivax*. The species of *Plasmodium* with very similar morphology, especially *P. vivax* and *P. ovale*, are difficult to differentiate using microscopy; however, the amplification of ssrDNA shows a fragment that is characteristic of *P. ovale*⁽⁸⁾.

In this pilot project, we used microscopy and molecular diagnosis to actively search for asymptomatic cases in Santa Teresa, a small indigenous locality with 440 inhabitants, located in the department of Caaguazú in the eastern region of Paraguay (Figure 1). The inhabitants mainly belong to the Mbya Guaraní ethnic group (92.7%), followed by the Ava Guaraní (7.0%) and Nivaclé ethnic groups (0.2%)⁽⁹⁾. In 2007, 30% of the population of this locality presented with infection of *P. vivax*. This study collaborated with the National Program for control of the disease (SENEPA) in order to identify asymptomatic cases that could pose a risk for the silent transmission of the malarial parasite.

Surveillance in the area is currently carried out by the SENEPA through diagnosis by microscopic examination of thick and thin blood smears. The SENEPA provides medicines free of charge and carries out follow-up of confirmed cases.

Based on these data, we identified a sample size of 102 to achieve 90% confidence for this cross-sectional descriptive study⁽¹⁰⁾.

Microscopic analysis was performed for Giemsa-stained thick and thin blood smears. Magnification under immersion oil was $\times 1000$, and slides were considered negative after examining 100 fields.

Blood from each participant included in this study was collected using filter paper (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) after a finger prick for molecular diagnosis and using microscopy slides to obtain thick and thin smears for microscopic examination. Each blood drop was dried at room temperature and each filter paper was stored at -20°C for DNA extraction. DNA was extracted from each blood drop in the filter paper using the Chelex method⁽¹¹⁾.

In order to identify the *Plasmodium* species, we performed SnM-PCR to amplify the small subunit of the 18S ribosomal gene (ssrDNA) of *Plasmodium*⁽¹²⁾. This gene is a multicopy gene, with conserved regions and characteristic regions for each of the following four common species that infect humans: *P. falciparum*, *P. ovale*, *P. vivax*, and *P. malariae*. In the second round, the amplification is performed using the PCR product from the first round as a template to amplify the four species of *Plasmodium* (*P. falciparum* 370 bp, *P. ovale* 407bp, *P. vivax* 476bp, and *P. malariae* 241bp)⁽¹²⁾. Details of the reaction have been described by del Puerto et al. in 2015⁽⁸⁾.

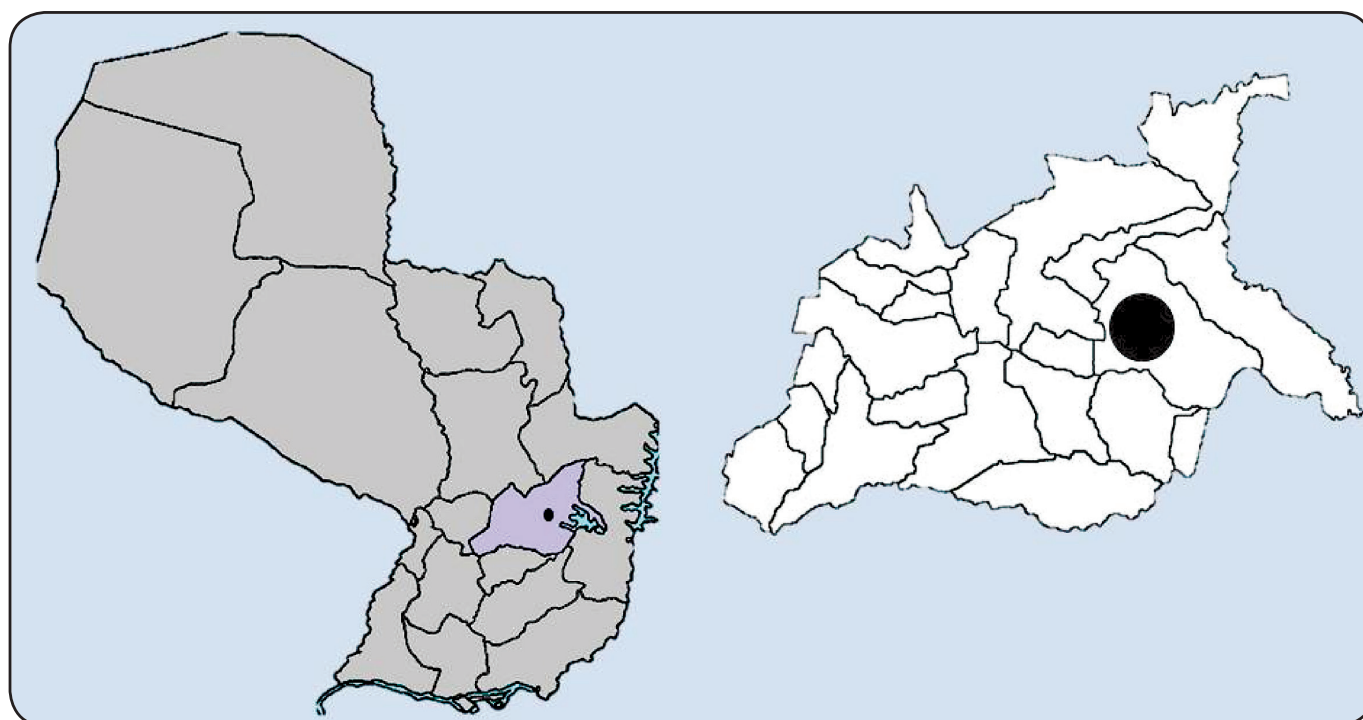


FIGURE 1. The locality of Santa Teresa in the map of Paraguay.

In both reactions, as positive control, we used genomic DNA of *P. falciparum* RO-33, MRA-200G, obtained through MR4 from the BEI Resources Repository, National Institute of Allergy and Infectious Diseases, and National Institute of Health. The PCR products were analyzed using 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized using UV light. The sizes of the bands were compared with a molecular weight marker of 100bp.

The present study was approved by the ethics review board of the Instituto de Investigaciones en Ciencias de la Salud, Universidad Nacional de Asunción (IICS-UNA, code P22/2015). Informed consent was obtained from each individual, the data were kept confidential, and the samples were coded for handling.

Random sampling was successfully performed in 100 individuals aged over 18 years from both sexes. Eighty-nine percent of the participants had a history of malaria. Of the 100 participants, 44 were female (mean age, 34 ± 16 years) and 56 were male (mean age, 40 ± 18 years) (Table 1). All participants belonged to the Mbya Guaraní ethnic group.

We found that neither the microscopic method nor the molecular method identified any parasite. Additionally, no characteristic bands for the genus *Plasmodium* or the four species of *Plasmodium* were observed in the 100 DNA samples analyzed. In contrast, we successfully amplified the human internal positive control (Table 1).

Plasmodium vivax predominates in countries that are the principal candidates for malaria elimination. Owing to the difficulties associated with the control of this species, its incidence has decreased more slowly than that of *P. falciparum* in the regions where both species co-exist. Hence, in the future, *P. vivax* could persist as the main cause of malaria and could be the main challenge for the elimination of the disease⁽²⁾.

The number of countries that have progressed toward the elimination of malaria is increasing. Paraguay is among these countries, and fortunately, along with 15 other countries, it reported zero indigenous cases in 2014⁽²⁾. Based on this situation, our country cannot economize control strategies considering that the indigenous parasite is *P. vivax*. The implementation of active surveillance of asymptomatic cases in historically endemic regions in Paraguay is needed.

Contrary to *Plasmodium falciparum*, *Plasmodium vivax* can remain latent as hypnozoites in the host. It is difficult to identify the intermittency period of the parasite, especially when the disease is asymptomatic. At this stage, the parasitemia is unnoticed. Therefore, individuals do not receive treatment and they become part of the reservoir. Considering the presence of *Anopheles* mosquitoes in the region, there is a local transmission risk.

To our knowledge, this is the first study on active surveillance for asymptomatic cases of malaria in Paraguay. The locality of Santa Teresa was a good model to establish active surveillance of asymptomatic cases using molecular methods because it has a small population and a history of high endemicity, which is reflected by the fact that 89% of the participants had a history of malaria, and this strengthens our results.

Epidemiological silence in our country began in 2012, and the last cases reported in Santa Teresa were from 2008, when 26 cases were recorded (unpublished data from SENEPA). The elimination of the disease requires diagnostic methods that are readily available and effective, and that allow the detection of subpatent parasitemia, with detection limits below those of the gold standard (i.e., 4-20 parasites/ μL ⁽¹³⁾) to verify local transmission. In countries with low endemicity, rapid diagnostic tests, which provide results in minutes, have disadvantages owing to the lack of sensitivity. Moreover, it has been shown that antimalarial antibodies disappeared in cases that were treated 8 years previously⁽¹⁴⁾.

The results obtained in this study are very promising, as no asymptomatic cases were observed using SnM-PCR or microscopy. Nonetheless, to affirm strongly that there are no more asymptomatic cases in this locality, a longitudinal study should be carried out. A similar study was performed in Iran, and the authors did not note any asymptomatic cases using either of the methods⁽¹⁵⁾. This previous study was based on the seasonality of malaria, as malaria shows the following two peaks in Iran: June-July and September-October. The authors performed three samplings between 2009 and 2010 (one after the first peak, another during the second peak, and the third after the second peak)⁽¹⁵⁾. Based on this approach, it would be interesting to determine the frequency and duration of sampling in a longitudinal study in Paraguay, according to the seasonality of malaria.

Seminested multiplex-polymerase chain reaction, as a molecular tool, was very useful as it allowed us to verify that there was no local transmission of the disease from human reservoirs, indicating the high potential of this locality to eradicate the disease in a short period. Seven years have passed since the last reported cases in the locality of Santa Teresa. However, we cannot lower our guard with regard to the ability to recognize clinical cases and perform microscopic analysis, considering that the locality is surrounded by localities of Brazilian migrants and that there is a risk of potential re-introduction of the disease.

Our next goal is to collect vectors from the same area and perform the same molecular analysis, as well as to expand the same study to another 10 historically endemic regions in order to generate enough information about the prevalence of

TABLE 1
Characteristics of the study participants.

Number = 100		
	Mean age (years)	Standard deviation
Microscopy (thin and thick smears)		
SnM-PCR		
Sex		
female	44	16
male	56	34
History of malaria		
yes		no
no	89	11

SnM-PCR: seminested multiplex-polymerase chain reaction.

asymptomatic cases in Paraguay. This will allow a better and deeper evaluation of the control of malaria in Paraguay.

In conclusion, there were no asymptomatic cases of malaria in Santa Teresa. We believe that molecular methods are appropriate for evaluating asymptomatic cases.

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Conflict of interest

The authors declare that there is no conflict of interest.

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