



## Concurrent dengue and malaria in the Amazon region

Co-infecção por dengue e malária na região Amazônica

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### ABSTRACT

**Introduction:** The Amazon region has extensive forested areas and natural ecosystems, providing favorable conditions for the existence of innumerable arboviruses. Over 200 arboviruses have been isolated in Brazil and about 40 are associated with human disease. Four out of 40 are considered to be of public health importance in Brazil: Dengue viruses (1-4), Oropouche, Mayaro and Yellow Fever. Along with these viruses, about 98% of the malaria cases are restricted to the Legal Amazon region. **Methods:** This study aimed to investigate the presence of arboviruses in 111 clinical serum samples from patients living in Novo Repartimento (Pará), Plácido de Castro (Acre), Porto Velho (Rondônia) and Oiapoque (Amapá). The viral RNA was extracted and RT-PCR was performed followed by a Multiplex-Nested-PCR, using Flavivirus, Alphavirus and Orthobunyavirus generic and species-specific primers. **Results:** Dengue virus serotype 2 was detected in two patients living in Novo Repartimento (Pará) that also presented active *Plasmodium vivax* infection. **Conclusions:** Despite scant data, this situation is likely to occur more frequently than detected in the Amazon region. Finally, it is important to remember that both diseases have similar clinical findings, thus the diagnosis could be made concomitantly for dengue and malaria in patients living or returning from areas where both diseases are endemic or during dengue outbreaks.

**Key-words:** Arboviruses. Coinfection. Flaviviruses. Dengue. Malaria. Amazon region.

### RESUMO

**Introdução:** A região Amazônica possui extensas áreas florestadas e ecossistemas naturais, provendo condições favoráveis para a existência de diversos arbovírus. Aproximadamente, 200 arbovírus foram isolados no Brasil, e 40 estão associados com doenças em humanos. Quatro destes 40 são considerados ser de importância para a saúde pública no Brasil: vírus da dengue (1-4), Oropouche, Mayaro e febre amarela. Juntamente com estes vírus, aproximadamente 98% dos casos de malária estão restritos à região da Amazônia Legal. **Métodos:** O objetivo deste estudo foi investigar a presença de arbovírus em 111 amostras clínicas de sangue de pacientes que residiam em Novo Repartimento (Pará), Plácido de Castro (Acre), Porto Velho (Rondônia) and Amapá (Macapá). O RNA viral foi extraído, RT-PCR foi realizada seguida de uma Multiplex-Nested-PCR, usando primers genéricos e espécie-específicos para Flavivirus, Alphavirus and Orthobunyavirus. **Resultados:** Detectamos o vírus da dengue, sorotipo 2, em dois pacientes que residiam em Novo Repartimento (Pará), que também tinham infecção por *Plasmodium vivax*. **Conclusões:** Apesar de dados escassos, esta situação, provavelmente, ocorre mais frequência que a detectada na região Amazônica. Definitivamente, é importante lembrar que ambas as doenças possuem achados clínicos similares, assim o diagnóstico deveria ser feito concomitantemente para dengue e malária em pacientes que residem ou estão voltando de áreas onde ambas as doenças são endêmicas.

**Palavras-chaves:** Arbovírus. Co-infecção. Flavivírus. Dengue. Malária. Região Amazônica.

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### INTRODUCTION

Every year, thousands cases of malaria are diagnosed and about 98% of them are restricted to the Legal Amazon region<sup>1</sup>. However, in the Brazilian Amazon region, approximately 200 arboviruses have been isolated and about 40 have been associated with human disease. Among these viruses, dengue viruses are the most important flavivirus causing human disease and outbreaks of important public health impact<sup>2</sup>.

Recently, concurrent dengue and malaria was reported in the Amazon region of French Guiana<sup>3</sup>. Altogether, there are few reports concerning dengue and malaria coinfection and all of them were isolated cases<sup>4-10</sup>.

In the Brazilian Amazon region, concomitant infection with malaria and arbovirus was reported in 1990, though the first detection was in 1971. Five other incidences were identified in 1974, 1983, 1984 and 1987. These cases of fever of unknown origin were previously diagnosed as malaria and despite treatment, the symptoms remained. The agents identified were Catu, Guaroa and Tacaiuma viruses, all belonging to the genus Orthobunyavirus, family *Bunyaviridae*<sup>11</sup>. Since the 1980s dengue has become a major public health issue in Brazil and has become more frequent in the Amazon region. Thus, the aim of this study was to investigate the presence of arboviruses (especially dengue virus) in clinical samples from patients with malaria residing in the Amazon region.

### METHODS

#### Study areas

Oiapoque (03°50'35" S; 51°50'06" W) is 560km from the capital of State of Amapá, and is located on the Amazon River within tropical forest. Its estimated population is 20,962 inhabitants and its Annual Parasitic Index (API) was 6.0 in 2009. Porto Velho (-8° 45' 43" S; 63° 54' 14" W) is the capital of State of Rondônia in the upper Amazon River basin, with 383,425 inhabitants and an API of 53.7 in 2009. Plácido de Castro (10° 16' 33" S; 67° 09' 00" W)

is located at the border of the country Bolivia as well as Rondonia and Amazonas States, it has a population of 18,235 inhabitants and its API was 20.6 in 2009. Novo Repartimento (04° 19' 50'' S; 49° 47' 47'' W) is a gold mining area in southeastern State of Pará. Its population is estimated at 55,759 habitants and it had an API of 15.4 in 2009. The climate in these areas is characterized as tropical with no dry season; the mean monthly precipitation level is at least 60mm.

#### Clinical samples

Male and female malaria patients (n=111) from four regions of the Brazilian Amazon were enrolled in this study: 40 samples from Novo Repartimento, 29 samples from Porto Velho and 30 samples from Plácido de Castro were obtained from May 2003 to August 2005. Additionally, 12 samples from the municipality of Oiapoque, State of Amapá were obtained in 2009 (Figure 1). These individuals sought attendance on their own initiative and were invited to participate in this study at the public healthcare clinics in each study area. They were all over 18 years-old and had positive thick blood film results for *P. falciparum* or *P. vivax* single infection. Pregnant women, patients under 18 years-old and those with no other concomitant illness were excluded from the study. Participants were asked to sign a written free informed consent form before blood samples were collected. The consent form was cosigned by a staff member of the clinic. Clinical and epidemiological data such as age, sex, past history of malaria and current infection information were obtained during a specific interview conducted by the physicians and also from medical records.

#### Clinical evaluation

All patients voluntarily sought medical assistance presenting with uncomplicated clinical malaria symptoms, as determined by the physicians and/or nurses enrolled in the malaria diagnosis and treatment routine of the Brazilian government national program. Individuals who presented at least one of the following symptoms:

fever, headache, and shivering, in addition to microscopic positivity, were included in the post diagnostic medical evaluation. Likewise, symptoms were defined as *present* or *absent* by the medical staff according to the temperature measurements performed by the nurses and also by a detailed, specific interview, regarding unusual and/or previously experienced clinical malaria manifestation. Of all the clinical aspects recorded during the 111 malaria attacks, a typical febrile paroxysm was the most frequent clinical symptom, observed in 97.4% of cases, as a single or an associated manifestation. Associations between the three clinical aspects assessed (fever, headache and shivering) showed fever plus headache in 85.2% of cases, while fever plus shivering was reported in 88.7%.

#### Study strategy

The sera were stored at -80°C. Viral RNA was extracted from 140µl of each serum with the QIAamp viral RNA Mini kit (QIAGEN, Inc.) in accordance with the manufacturer's instructions and yielded a final volume of 60µl.

Initially, clinical samples were tested for the presence of flavivirus and alphavirus, as described by Bronzoni et al<sup>12</sup>. A Duplex-RT-PCR (D-RT-PCR) was performed using Flavivirus and Alphavirus universal primers simultaneously. After, three Multiplex-Nested-PCR (M-N-PCR) were performed using specie-specific primers to detect and identify dengue virus (DENV1-3), yellow fever virus (YFV) and Saint Louis Encephalitis virus (SLEV); another to detect DENV-4, Rocio virus (ROCV), Ilhéus virus (ILHV) and West Nile virus (WNV); and a third to detect Eastern Equine Encephalitis virus (EEEV), Venezuelan Equine Encephalitis virus (VEEV), Western Equine Encephalitis virus (WEEV), Mayaro virus (MAYV) and Aura virus (AURAV). Finally, a RT-PCR with primers that anneal to the 5' and 3' extremities of the genome segments of bunyaviruses and internal primers that anneal to the S segment of Simbu serogroup viruses in a Nested PCR was used to amplify the Oropouche virus



FIGURE 1 - Study area where samples were collected in the Amazon region of Brazil, Macapá, Amapá State (AP; 00° 02' 20'' S; 51° 03' 59'' W); Novo Repartimento, State of Pará (PA; 04° 19' 50'' S; 49° 47' 47'' W); Porto Velho, State of Rondônia (RO; 08° 45' 43'' S; 63° 54' 14'' W) and Plácido de Castro, State of Acre (AC; 10° 16' 33'' S; 67° 09' 00'' W).

genome, as described by Morelli et al<sup>13</sup>. The amplicons were loaded into a 1% agarose electrophoresis gel and visualized under ultraviolet light. Amplicon sizes were determined by comparison with a 100bp DNA ladder (Promega). Precautions to avoid contamination were followed; positive and negative controls were used in all reactions. All amplicons were also sequenced in order to confirm the specificity of the amplification using the PCR primers Big Dye v3.1 (Applied Biosystems, CA) in a ABI3130 automated sequencer.

### Ethical

The protocol for this study was reviewed and approved by the Research Board of the Faculty of Medicine from São José do Rio Preto.

## RESULTS

Of the total of 111 malaria-positive patients that were tested for arboviral detection, 80 presented *Plasmodium vivax* and 31 presented *Plasmodium falciparum* infections. Concurrent dengue virus serotype 2 and malaria were confirmed in 2 of the 111 patients (Figure 2), who possessed prior laboratorial diagnosis of malaria before molecular screening for arboviruses. These patients were a 57 year-old male and a 20 year-old female living in Novo Repartimento (Pará) and both had active *Plasmodium vivax* infections.

Virus isolation was attempted but was unsuccessful, because it is very difficult to isolate arboviruses and the blood samples have to be collected when the viremia is occurring, which means during the first 5 days of the disease.

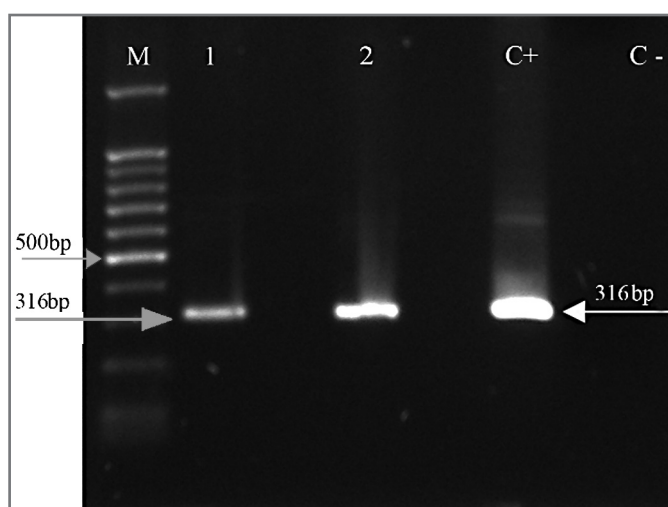


FIGURE 2 - Shows the amplifications obtained for DENV-2 (316 bp). M: molecular marker; lanes 1 and 2: samples positive for DENV-2; C+: positive control; C-: negative control. The fragments were purified from PCR mixtures and sequenced using the BigDye v3.1 Terminator (Applied Biosystems, Foster City, CA, USA) and an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences were analyzed using the DS Gene 2.0 Software (Accelrys, USA) and were confirmed as DENV-2.

## DISCUSSION

The Brazilian Amazon region has extensive forested areas, natural ecosystems and a large diversity of animals that provide a suitable environment for arbovirus transmission. Currently, 99.8% percent of malaria cases are restricted to the Legal Amazon region<sup>1</sup>, where

approximately 40 arboviruses considered to be of importance to public health cocirculate<sup>14</sup>.

In Brazil, 54 species of *Anopheles* mosquitoes are currently known and 33 of these occur in the Brazilian Amazon region<sup>15,16</sup>. *An darlingi*, *An nuneztovari* and *An albitarsis* are considered of special interest, because they have been detected with *Plasmodium* infection near human habitations<sup>15</sup>. Nowadays, *An darlingi* is the main vector, because it is highly anthropophilic. *An nuneztovari*, *An triannulatus* and *An albitarsis* are considered to be secondary vectors, since they are detected with *Plasmodium* infection only in areas where *An darlingi* initiated an outbreak<sup>16</sup>.

However, some arboviruses isolated in the Amazon region have *Anopheles* sp mosquitoes as their vectors, including: the widely spread Guaroa virus that is associated with sporadic cases in the rural area, isolated from *An triannulatus* and *An nuneztovari*; and Tacaiuma virus, associated with sporadic cases in enzootic foci, isolated from *An Triannulatus*<sup>17</sup>.

Despite these reports, the concomitance of dengue and malaria reported here is merely incidental, because this arbovirus has *Aedes aegypti* and *Aedes albopictus* mosquitoes as vectors<sup>18</sup>, which are not considered to be vectors of *Plasmodium* sp protozoans.

Mixed infections with many etiologic agents are not uncommon in malaria<sup>19</sup>. Despite scant data, dengue and malaria coinfection should be common in areas where both diseases are co-endemic in many places of the world<sup>10</sup>. In the Amazon region of Brazil, this situation is likely to occur more frequently than detected, considering that malaria is the most prevalent disease<sup>1</sup> and dengue is endemic to this region as well<sup>20</sup>. In this context, a coinfection with malaria and dengue cannot be ruled out and should be suspected in patients living or returning from areas where both diseases are endemic.

The most common clinical findings for dengue are fever (100%), myalgia (79%), rash (79%), headache (68%), nausea (37%) and diarrhea (37%)<sup>21</sup>. Similarly, the typical clinical findings for malaria are high-grade fever followed by chills, profuse sweating and headache that occur intermittently depending on the infecting *Plasmodium* species. However, in some patients prodromal symptoms appear some days before disease paroxysms, including nausea, vomiting, asthenia, fatigue and anorexia<sup>1</sup>. In the Brazilian Amazon region, malaria predominates in Mesoendemic conditions with wide variations in transmission, as can be observed by the nonimmune or semi-immune status of the adult population, as well as by asymptomatic carriers. An explanation for the reduction in symptoms in mixed infection carriers could be the mean age of the affected patients and time of residence in the endemic area, since it is well documented by different authors that immunity can play an important role in malaria symptom relief<sup>22,23</sup>. Consequently, minor clinical evidence for malaria was noted by the patients studied once a reduction in the severity of malaria symptoms was reported in individuals with limited preexposure to different species<sup>24</sup>. Nevertheless, it is not a general consensus that higher fevers, per se, are the consequence of greater clinical severity or more effective immune response<sup>25</sup>.

In a recent study regarding diagnostic techniques and management of dengue and malaria coinfection, all patients with dual infection presented prolonged fever for more than seven days, myalgia, bleeding manifestations, rash and anemia<sup>26</sup>. Moreover, according to Vasconcelos et al, the continuous fever caused by arboviral infection can mask the periodic fever associated with malarial parasites<sup>11</sup>.

Although a reduced sample number was assessed in this study, a limitation that we acknowledge, it is important to remember that dengue and malaria coinfection requires special attention because delayed diagnosis and appropriated treatment can result in fatal complications<sup>6,10</sup>. Both diseases cause similar symptoms and simultaneous infections with two different infectious agents may result in overlapped symptoms<sup>4</sup>; diagnosis of malaria and dengue based purely on clinical grounds may become difficult for physicians<sup>10</sup> and it is possible that either clinical spectrum of the disease or treatment may also be affected<sup>9</sup>. Finally, it is important to remember that both diseases have similar clinical findings, thus the diagnosis could be made concomitantly for dengue and malaria in patients living or returning from areas where both diseases are endemic or during dengue outbreaks.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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