

Epidemiological profile of Zika, Dengue and Chikungunya virus infections identified by medical and molecular evaluations in Rondonia, Brazil

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ABSTRACT

Several arboviruses have emerged and/or re-emerged in North, Central and South-American countries. Viruses from some regions of Africa and Asia, such as the Zika and Chikungunya virus have been introduced in new continents causing major public health problems. The aim of this study was to investigate the presence of RNA from Zika, Dengue and Chikungunya viruses in symptomatic patients from Rondonia, where the epidemiological profile is still little known, by one-step real-time RT-PCR. The main clinical signs and symptoms were fever (51.2%), headache (78%), chills (6.1%), pruritus (12.2%), exanthema (20.1%), arthralgia (35.3%), myalgia (26.8%) and retro-orbital pain (19.5%). Serum from 164 symptomatic patients were collected and tested for RNA of Zika, Dengue types 1 to 4 and Chikungunya viruses, in addition to antibodies against Dengue NS1 antigen. Direct microscopy for Malaria was also performed. Only ZIKV RNA was detected in 4.3% of the patients, and in the remaining 95.7% of the patients RNA for Zika, Dengue and Chikungunya viruses were not detected. This finding is intriguing as the region has been endemic for Dengue for a long time and more recently for Chikungunya virus as well. The results indicated that medical and molecular parameters obtained were suitable to describe the first report of symptomatic Zika infections in this region. Furthermore, the low rate of detection, compared to clinical signs and symptoms as the solely diagnosis criteria, suggests that molecular assays for detection of viruses or other pathogens that cause similar symptoms should be used and the corresponding diseases could be included in the compulsory notification list.

KEYWORDS: Zika. Chikungunya. Dengue. One-step real-time RT-PCR. Diagnostic. Arboviruses.

INTRODUCTION

Climate conditions together with large populations of mosquitoes from the genera *Aedes* sp. and *Anopheles* sp. in the Western Brazilian Amazon lead to the perfect environment for the maintenance of arboviruses and protozoa transmission cycles. Outbreaks of Dengue virus (DENV), Yellow Fever virus (YFV), Oropouche virus (OROV), Mayaro virus (MAYV) and the *Plasmodium* sp. protozoa parasites have already been reported in this region¹⁻⁴.

The Zika virus (ZIKV) was first isolated in 1947 from a monkey in Uganda and sporadic infections were subsequently identified in humans. In 2007, the virus was

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found to be widespread among patients presenting with flu-like symptomatology during an outbreak in the Yap Island of the Federated States of Micronesia. The subsequent increased incidence of patients with microcephaly or Guillain-Barré Syndrome in Brazil has been intensively investigated and the resulting data have established an association between ZIKV infections and neurological disorders⁵⁻⁷.

Infection with the DENV normally leads to fever and flu-like signs and symptoms, but can also be fatal in some cases^{8,9}. Based on the epidemiological investigations reported to the World Health Organization, an estimated 50-100 million cases occur annually in more than 100 endemic countries, which indicates that half of the world's population is at risk⁹.

Chikungunya virus (CHIKV) infection was first reported in 1952 in Southern Tanzania. Chikungunya fever is characterized by joint pains and high fever. The virus has recently arrived in Brazil (2014), and from the first report to the second half of 2017, approximately 300,000 cases have been reported, and 40% of Brazilian municipalities had confirmed cases^{10,11}.

Vector-borne diseases have been a major challenge for public health, with mosquitoes being the main vectors of hundreds of microorganisms around the world¹². The ecological and environmental conditions of the Amazon region favor the abundant presence of several species, including those that are potential vectors of human pathogens¹³.

Arboviruses have been a significant and constant threat due to the ease of adaptation of these viruses to new environments and their capacity to infect both vertebrates and invertebrate hosts. However, the anthropogenic environmental factors, such as disorganized urbanization, population displacement and the precariousness of basic sanitation, have favored the transmission and spread of these viruses¹⁴.

The aim of this study was to investigate the presence of main arboviruses spreading in Brazil (ZIKV, DENV and CHIKV) in patients with symptoms suggestive of arboviral disease, who lived in Rondonia during the first half of 2017. In summary, to date, this study provided the first epidemiological data on patients in this region, in whom RNA from ZIKV were identified. It is suggested that different protocols for the diagnosis of arbovirus infections should be combined in order to develop an improved algorithm to identify specific pathogens.

MATERIAL AND METHODS

Study location

The study was developed at the Ambulatory of Malaria

Outpatient Center for Tropical Medicine (CEPEM), located at BR 364, Km 3.5 in the city of Porto Velho, an agency linked to the State Department of Health (SESAU). The outpatient clinic is a reference center in malaria, attending hundreds of people with characteristic symptoms of the infection.

Samples

This study was approved by the local Research Ethics Committee (1.474.102CEP/2016/CEPEM). Whole blood samples were collected during the first half of 2017. For this study, serum samples from symptomatic patients were used and there was no laboratory evidence of the presence of *Plasmodium* sp. according to the direct microscopy investigation. In the first moment, a medical consultation was carried out to investigate the clinical data and identify the symptoms in these patients. Individuals over 12 years of age and both sexes, whose symptoms had a clinical course of up to five days, were included. The participant must also be agreed to receive periodic telephone calls and other electronic media contacts. Exclusion criteria were patients who do not have samples for laboratory and immunological characterization, indigenous patients and patients from other vulnerable groups.

RNA extraction

Viral RNA was extracted from 140 µL of serum samples by the QIAamp® Viral RNA Mini Kit (QIAGEN, Germany), according to the manufacturer's instructions. RNA was eluted in 60 µL of the AVE buffer.

Real-time RT-PCR

The RNA samples were tested for the presence of ZIKV; DENV serotypes 1, 2, 3, and 4; and CHIKV by a one-step real-time RT-PCR Kit (Biomol ZDC, Instituto de Biologia Molecular do Parana, Brazil), following the manufacturer's instructions. Reactions were performed in an Applied Biosystems 7500 Real-Time PCR apparatus, as follows: 51 °C for 30 min, 95 °C for 15 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Reactions were considered positive when the threshold cycle was ≤ 36 for the virus analyzed and ≤ 28 for the internal control.

DENV NS1 antigen testing

Serum samples were tested using the Platelia DENV NS1 AG (BIO-RAD, USA), an enzyme immunoassay for qualitative or semiquantitative detection of the NS1 antigen in human serum or plasma.

Direct microscopy for malaria

Thick blood smears were stained with Giemsa which is the gold standard method for malaria diagnosis. Each blood smear was examined for the presence of *Plasmodium* by experienced technicians.

Statistical analysis

Data were entered into a database in Excel. The prevalences were analyzed with the WinEpi on-line package (Universidad de Zaragoza, Spain) and were presented as absolute values, with 95% confidence intervals (95% CIs). All statistical analyses were performed using the Prism® 7.01 software for Windows™ (GraphPad Software, San Diego, CA). In all cases, p values < 0.05 were considered statistically significant.

RESULTS

A total of 164 samples were collected during the first half of 2017, from patients whose symptoms were mostly self-declared. During the anamnesis, the main relevant signs and symptoms reported by patients were: headache 78/164 (78%), fever 84/164 (51.2%), arthralgia 58/164 (35.3%), myalgia 44/164 (26.8%), exanthema 33/164 (20.1%), retro-orbital pain 32/164 (19.5%), pruritus 20/164 (12.2%), chills 10/164 (6.1%) and conjunctivitis 7/164 (4.3%). Correlations between signs and symptoms of ZIKV-positive or negative patients are shown in Table 1.

Among the signs and symptoms reported by patients with viremia, only conjunctivitis had a statistical relevance (OR=12.16, 95% CI, 1.88-78.63, p=0.03), with a positive correlation between conjunctivitis and positivity for ZIKV, although only two cases had conjunctivitis.

RNA from the serum samples of 164 patients were tested by real-time RT-PCR. Amplification results revealed that 7 (4.3%) samples were positive only for ZIKV, and 157 were negative for all DENV serotypes, CHIKV and ZIKV viruses. Negative real-time RT-PCR results were validated by the internal control (Figure 1). All RNA samples amplified the internal control sequence (an endogenous human transcript), ensuring that these samples were not degraded and that the negative results were true negatives. The serological test for NS1 DENV antigen was negative for all serum samples. The microscopic evaluation of 164 peripheral blood smears was negative for malaria infection. In addition, the specificity of the real-time RT-PCR assay (Kit Biomol ZDC) for ZIKV, DENV and CHIKV was assessed by testing samples known to be positive for *Plasmodium falciparum*; MAYV, YFV and OROV; as well as ZIKV, DENV1, DENV2, DENV3, DENV4 and CHIKV. Data showed specific amplifications only, confirming the absence of cross-reactivity with the other evaluated pathogens. Assays were validated by the positive control supplied by the manufacturer (Figure 2).

DISCUSSION

Infections caused by arboviruses and *Plasmodium* sp. occur in the same geographical regions due to the association between the climate and the presence of the vectors *Aedes* sp. and *Anopheles* sp., respectively. Patients with these infections present similar signs and symptoms, including chills, fever and headache, which may be mild and difficult to recognize^{1,12-14}. In this study, considering the signs and symptoms reported by patients with viremia, only conjunctivitis was more common in ZIKV-positive cases, with 28.6% of patients with ZIKV infection presenting the symptom, corroborating results described in the literature^{15,16}. Although conjunctivitis is reported in cases

Table 1 - Signs and symptoms reported by patients.

Signs and symptoms	Positive ZIKV		Negative ZIKV		p value	OR (95% CI)
	n=7	(%)	n=157	(%)		
Fever	5	71.4	79	50.3	0.274	2.47 (0.46 -13.11)
Arthralgia	4	57.1	54	34.4	0.245	2.54 (0.55-11.78)
Myalgia	3	42.9	41	26.1	0.387	2.12 (0.45-9.89)
Exanthema	3	42.9	30	19.1	0.146	3.17 (0.67-14.95)
Itching	2	28.6	18	11.5	0.204	3.09 (0.56-17.12)
Retro-Orbital Pain	3	42.9	29	18.5	0.135	3.31 (0.70-15.61)
Headache	3	42.9	75	47.8	1.000	0.82 (0.18-3.79)
Chills	0	0.0	10	6.4	1.000	0.94 (0.05-17.56)
Conjunctivitis	2	28.6	5	3.2	0.030	12.16 (1.88-78.63)

OR - Odds ratio; CI - Confidence interval.

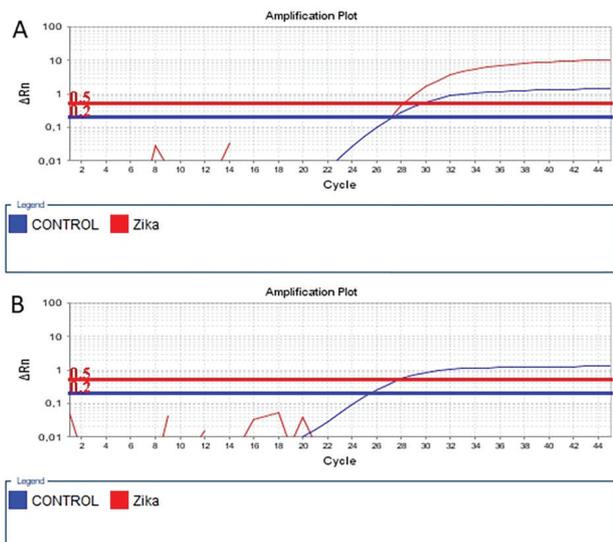


Figure 1 - Amplification profile depicting the threshold cycles (Cts) of positive patients and negative ones: A) Red line: positive ZIKV patient; B) Blue line: a negative patient to ZIKV, showing only the amplification of the internal control.

of ZIKV, it is not pathognomonic of this infection, as it has already been described in cases of DENV infection¹⁶, and has been observed in this study in 3.2% of ZIKV, DENV and CHIKV-negative cases, in infections probably caused by other pathogens.

One hundred and sixty-four patients presenting with symptomatology suggestive of arboviral infections were evaluated by medical, molecular and microscopic methods and, in this study, the first cases of symptomatic ZIKV infections were identified in Rondonia State, by a one-step

real-time RT-PCR assay, after a worldwide explosive outbreak of the virus in 2015.

The Epidemiological Bulletin from the Secretariat of Health Surveillance, Ministry of Health¹⁷, reported that the DENV prevalence in Rondonia State during 2017, based only on clinical signs and symptoms, was of 1,807 cases. For ZIKV and CHIKV, the numbers were 101 and 163, respectively. However, in this study, the detection rate of viral ZIKV RNA was 4.3% in the same region. Thus, our results suggest that the notification based only on epidemiological criteria and clinical symptomatology is unsuitable as diagnoses are not always confirmed by laboratory tests. Our cohort was composed of only symptomatic patients, and the number of ZIKV confirmed cases was low. To circumvent this problem, we suggest the use of molecular diagnostic protocols to assess the etiologic agents of arboviruses and other pathogens circulating in Brazil.

The nucleic acid assay ZDC BioMol kit (IBMP) used to detect RNA from ZIKV, DENV and CHIKV was effective in a previous epidemiological study, using different types of biological specimens such as urine and saliva samples, demonstrating that is an important tool for identification and differentiation of these arboviruses¹⁸.

Several surveillance strategies have been carried out for ZIKV, DENV and CHIKV infections in some localities in Northern Brazil. However, no screening study for ZIKV had been previously performed in Rondonia State. Currently, there are ongoing surveys on DENV and more recently on CHIKV fever (e.g., first description of CHIKV in Amapa State)¹⁹.

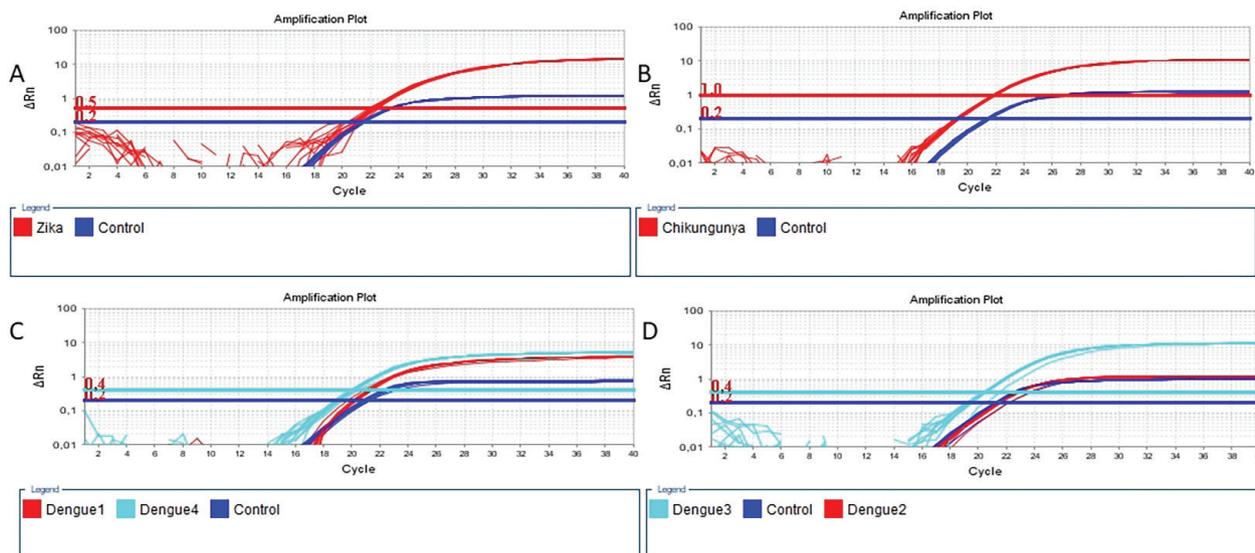


Figure 2 - Amplification profiles depicting the Cts (on the abscissa axis) of positive samples for ZIKV, CHIKV, and DENV and the controls supplied by the Kit Biomol ZDC: A) Zika; B) Chikungunya; C) Dengue 1 and Dengue 4; D) Dengue 2 and Dengue 3. The amplification profiles of the studied viruses are represented in red or turquoise, while the controls are always represented in blue. The emitted fluorescence threshold (on the ordinate axis) for Zika is 0.5; for Dengue 1 and the Control is 0.2; for Dengue 2 is 0.5 and for Dengue 3 and 4 is 0.4 and for Chikungunya is 1.

The time lapse from the first molecular detection of ZIKV in Rondonia State to the first reported detection of autochthonous ZIKV in Brazil²⁰ suggests that this virus is in the process of spreading in this region, as previously described for DENV and CHIKV.

The small number of studies, together with the difficulty of identifying ZIKV, may be associated with geographic characteristics of the region. In addition, the co-circulation of other arboviruses, such as DENV, CHIKV, MAYV and OROV, which have overlapping symptoms to ZIKV infection (low fever, pruritus and cutaneous rash), may pose an additional challenge to understand the epidemiology of ZIKV infection in Rondonia State. Furthermore, the literature describes that the percentage of Zika asymptomatic or mild symptomatic cases is approximately 80% and this factor could be associated with our symptomatic cohort^{21,22}.

It is worth noting that DENV was not detected in this study, even by serological and molecular methods and considering that the region is endemic to this virus²³.

Our new findings are of paramount importance to public health authorities, as ZIKV infections, as observed mainly in the Northeast region, has been responsible for the occurrence of complicated neurological syndromes in Brazil²⁴. Due to the dispersion of specific ZIKV vectors throughout the country, in addition to the other major arboviruses, molecular screening should be considered as an important tool for monitoring patients with acute febrile diseases. Finally, the low rate of detection of ZIKV-infected individuals (7/164) and the absence of positive cases for DENV, CHIKV and *Plasmodium* sp. infections suggest that other pathogens have been circulating in this area. Because several outbreaks of arboviruses infections in the Rondonia have been reported, especially of DENV²³, medical and molecular investigations for new arboviruses that are spreading throughout this region are needed. The list of new viruses should include the West Nile virus (WNV), MAYV, YFV and OROV.

CONCLUSION

To the best of our knowledge, this study described the first molecular evidence of human symptomatic ZIKV infections in Rondonia, a region considered highly endemic for arboviruses. As the signs and symptoms presented by the investigated population are similar to those of other arboviruses, an accurate laboratory scenario for the molecular screening of the main circulating arboviruses in our region is of prime importance.

CONFLICT OF INTERESTS

The authors declare no potential conflict of interest.

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