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Detection of coinfection with Chikungunya virus and Dengue virus serotype 2 in serum samples of patients in State of Tocantins, Brazil

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ABSTRACT

Background: The co-circulation of Chikungunya (CHIKV), Dengue (DENV) and Zika (ZIKV) viruses increased the risk of outbreaks and coinfections among them. Here, we report cases of coinfection in clinical samples from state of Tocantins, Brazil.
Methods: In 2017, the Central Public Health Laboratory (LACEN) received samples of patients who consulted health units with symptoms compatible with arboviral infections. A total of 102 samples were sent to the Retrovirology Laboratory at the Federal University of São Paulo, where they were tested by RT-qPCR to confirm DENV, ZIKV and CHIKV infections and to detect coinfected patients.
Results: We identified with CHIKV monoinfection (52), DENV serotypes 1 (28) and serotypes 2 (22). We did not detect ZIKV. Five patients were characterized with coinfection involving CHIKV and DENV serotype 2.
Conclusions: The presence of co-circulating arboviruses increases the chance of coinfection and demonstrates the importance of differential diagnosis and vector control.

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Introduction

Cases of simultaneous infections involving different arboviruses are becoming common in areas where they circulate concomitantly. Vector density and environmental changes, along with migration and immigration contribute to the spread of these viruses. They are endemic in the tropical and subtropical regions of the world and are the main causative agents of infectious diseases of importance in public health [1].

Dengue virus (DENV) and Chikungunya virus (CHIKV) are endemic arboviruses in Brazil. DENV belongs to the family *Flaviviridae* (genus *Flavivirus*). There are four DENV strains known as DENV1 – 4 serotypes. CHIKV belongs to the family *Togaviridae* (genus *Alphavirus*); three different genotypes have been identified: West Africa, East-Central South Africa (ECSA) and the Asian genotype [2]. Both viruses are transmitted to humans by the *Aedes aegypti* and *Aedes albopictus* mosquitoes [3]. During the acute phase of infection, they can cause nonspecific febrile syndromes that can aggravate severe and debilitating clinical conditions [4].

The first Dengue epidemic in Brazil occurred in 1981 in the state of Roraima. Subsequently, outbreaks were reported in all regions of the country [5]. In Brazil, CHIKV is emerging and was first reported in 2014. The Asian and East-Central South African (ECSA) genotypes entered the northern (Amapá state) and northeast (Bahia state) regions, respectively. Currently, both genotypes circulate throughout the country [6].

The first case of Chikungunya in the Americas was reported in Saint Martin in 2013 and coincided with an epidemic Dengue on the island, resulting in the first cases related of DENV/CHIKV coin-

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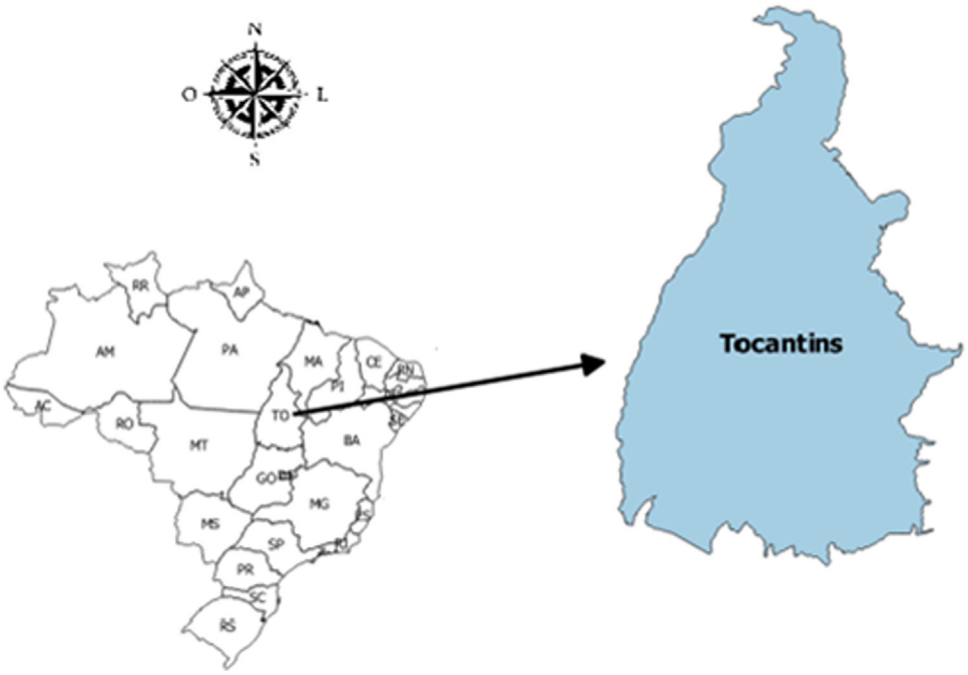

Fig. 1. Location of the state of Tocantins in Brazil. Limits with states of Goias (South), Piaui (East), Maranhao (Northeast), Bahia (Southeast), Para (Northwest) and Mato Grosso (Southwest). This map was built using the program QGIS, v. 2.18.

fection in the Americas [7]. Some studies have documented cases of patients coinfected with these viruses during some epidemics in South America. In a recent study conducted during a ZIKV epidemic in Colombia, of a total of 23,871 samples analyzed, 3 were reported to be coinfected with DENV/CHIKV [8]. Another study showed the co-circulation and coinfection of DENV, ZIKV and CHIKV in patients with febrile symptoms on the Colombian-Venezuelan border, where the prevalence of coinfection with DENV/CHIKV was 7.64% [9].

In Brazil, the co-circulation of these arboviruses was demonstrated and evidence of coinfections among them reinforce the need for differential diagnosis [10]. One project previously performed in Tocantins/Brazil demonstrated the co-circulation of different arboviruses in the state during an outbreak of ZIKV infection. In this work, 2 patients who showed symptoms of acute febrile illness were coinfected with DENV and CHIKV [11].

Simultaneous circulation of different arboviruses has caused frequent cases of coinfected patients during outbreaks or epidemics involving these viruses. In Brazil and many other tropical and subtropical countries where the arboviruses co-circulate, available information on coinfected individuals is scarce. The objective of our work was to confirm the presence of DENV, CHIKV and ZIKV at a molecular level and to detect coinfections in clinical samples from state of Tocantins, Brazil.

Methods

Study design

This cross-sectional observational study was realized with serum samples obtained from patients who consulted health units in the state of Tocantins with symptoms of arboviral infections. The samples were sent to the Central Public Health Laboratory (LACEN). The LACEN received a total of 450 samples. Of this total, 102 were sent to Laboratory of Retrovirology of the Federal University of São Paulo because they had a sufficient volume to permit a molecular analysis. The remaining 348 samples were not analyzed in this study because they are stored in LACEN and were not sent to the Retrovirology Laboratory. The symptoms of the patients involved in this study were described by the Tocantins health units and sent to the LACEN. Afterwards, these data were sent to the Retrovirology Laboratory.

Investigation region

Tocantins is an state Brazilian located in the northern region of country, bordering the states Goias (South), Piaui (East), Maranhao (Northeast), Bahia (Southeast), Para (Northwest) and Mato Grosso (Southwest) (Fig. 1). According to the Brazilian Institute of Geography and Statistics (IBGE), in 2017, the state had an estimated population of 1.555.229 million inhabitants and a demographic composition of 79% urban and 31% rural residents. Its human development index is 0.699 [12].

The Secretary of Health of the Tocantins reported an increase in the number of cases of infection by ZIKV in 2016, sporadic outbreaks of DENV between 2015–2017 and a outbreak of CHIKV in 2017 [13]. This shows that these viruses circulate simultaneously in the state. Thus, we looked for coinfections involving these arboviruses. All samples analyzed in this study were collected between the months of January to August of 2017.

Ethics statement

This study was approved by the Research Ethics Committee of the Federal University of Sao Paulo (CAAE: 18908719.2.0000.5505). All patients enrolled signed an informed written consent. The patients' personal information was anonymized before the data was accessed. This study accessed the information of the patients on demographic characteristics, clinical signs and symptoms.

Inclusion criteria

The study participants were people with more than 18 years of age, included both sexes and presented compatible symptoms of arboviral infections (fever, arthralgia, exanthema, headache, myalgia, nausea, retro-orbital pain and generalized body pain) and were

Table 1
Clinical characteristics of the five patients detected with coinfection in Tocantins, Brazil.

Patients[a]	Age (years) and gender	City	Symptoms of arboviral illness
1	46, male Araguaina		Fever, arthralgia, exanthema, nausea, conjunctivitis, retro-orbital pain, headache, leukopenia, myalgia.
2	19, female Palmas		Fever, arthralgia, exanthema, nausea, conjunctivitis, retro-orbital pain, headache, leukopenia, abdominal pain, vomits.
3	31, female Palmas		Fever, arthralgia, exanthema, nausea, conjunctivitis, retro-orbital pain, headache, leukopenia, abdominal pain, vomits.
4	34, male Araguaina		Fever, arthralgia, exanthema, nausea, myalgia, conjunctivitis, retro-orbital pain, headache, leukopenia, myalgia.
5	37, female Araguaina		Fever, arthralgia, exanthema, nausea, vomits, conjunctivitis, retro-orbital pain, headache, leukopenia, abdominal pain, myalgia.

[a] Relationship between age, sex, resident cities and clinical symptoms of coinfected patients.

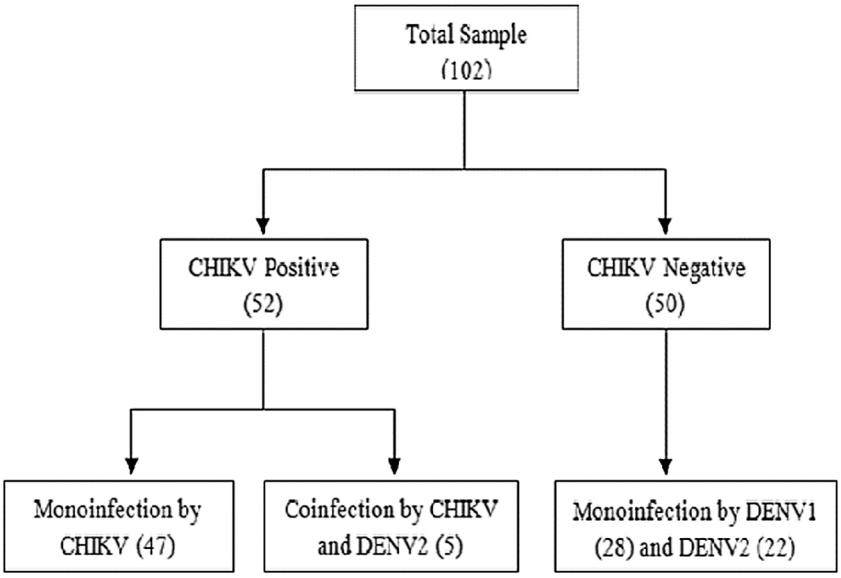

Fig. 2. Arbovirus detected in serum samples from patient in state of Tocantins, Brazil.

tested within 8 days of the onset of symptoms, following criteria established by the World Health Organization (WHO) [14] and Centers for Disease Control and Prevention (CDC) [15].

Molecular analysis

The 102 samples were analyzed by molecular diagnosis. We used the protocol of RT-qPCR established by Alm et al. [16] for serotyping Dengue virus in clinical samples. The protocols used to screening for ZIKV and CHIKV infection were established by Lanciotti el al [17] and Cecilia et al. [18], respectively. All samples were first tested for CHIKV, then analyzed for DENV and ZIKV.

First, viral RNA was extracted using the QIAamp Viral RNA Mini kit (Qiagen) following the manufacturer's instructions. The RT-PCR assay for detection of CHIKV, four serotype DENV and ZIKV was realized using the GoTaq® Probe1-Step RT-qPCR System (Promega) following recommendations of the manufacturer. The primers and probes used in this study were previously described [16–18]. The reaction was carried out using an ABI 7500 Real Time PCR system. The processing of all samples included negative and positive controls as well as internal controls (Ribonuclease P, RNAse P) to ensure reliability of the reaction. We did not perform serological tests in this study.

Statistical analysis

The mean age of the patients, the mean Cycling threshold (Ct) and the percentage of women and men were determined. The prevalence of monoinfection and coinfection by Chikungunya and Dengue were presented as absolute values with 95% confidence intervals. All statistical analyses were performed using Prism 7.01 for Windows TM.

Results

Of the 102 samples, 52 (cases) were confirmed as CHIKV positive and of this total 5 (cases) were identified with coinfection by DENV serotype 2. Clinical characteristics are in Table 1. The other 47 (cases) were characterized with monoinfection by CHIKV. Of the 50 (cases) that were diagnosed as CHIKV negative, we detected monoinfection by Dengue virus serotype 1 (28 cases) and 2 (22 cases), as illustrated in Fig. 2. We observed a prevalence of 46.07% (95% CI: 37.13–56.86) patients confirmed with monoinfection by CHIKV and 4.90% (95% CI: 0.72–9.27) coinfected with Dengue virus serotype 2. The results of the serotyping by multiplex PCR showed that 27.45% (95% CI: 19.16–36.83) suffered monoinfection by Dengue virus serotype 1 and 21.56% (95% CI: 13.85–30.14) monoinfection by Dengue virus serotype 2.

We used a positive control for CHIKV kindly provided by Dr. Ricardo Khouri and his team from Gonçalo Moniz Institute, Laboratory of Vector-borne Infectious Diseases, Fiocruz, BA. In addition, internal control for all reactions was used. The time between symptom onset and collection of all these samples varied from 4 to 7 days. The literature says that samples collected during the first week after symptom onset should be tested by serological and molecular methods (RT-PCR) and during the first 8 days after the onset

Table 2
Signs and symptoms presented by DENV or CHIKV monoinfected patients.

Signs and Symptoms	Monoinfection (% Signs and symptoms)		
	CHIKV (n = 47)	DENV-1 (n = 28)	DENV-2 (n = 22)
Fever	42 (89.3)	23 (82.1)	18 (81.8)
Exanthema	18 (38.2)	25 (89.2)	17 (77.2)
Headaches	37 (78.7)	22 (78.5)	17 (77.2)
Arthralgia	43 (91.4)	19 (67.8)	15 (68.1)
Myalgia	34 (72.3)	24 (85.7)	19 (86.3)
Diarrhea	6 (12.7)	4 (14.2)	2 (9.09)
Non-purulent conjunctivitis	14 (29.7)	3 (10.7)	1 (4.54)
Retro-orbital pain	10 (21.2)	28 (100)	22 (100)
Abdominal pain	5 (10.6)	20 (71.4)	16 (72.7)
Edema	4 (8.5)	0	0
Hemorrhagic manifestations	0	19 (67.8)	13(59.0)
Generalized body pain	44 (93.6)	28 (100)	21 (95.4)
Nausea	17 (36.1)	15 (53.5)	17 (77.2)
Vomiting	14 (29.7)	13 (46.4)	12 (54.5)
Leukopenia	11 (23.4)	9 (32.1)	7 (31.8)

of symptoms, CHIKV viral RNA can often be identified in serum [13,14]. This demonstrates the reliability of these negative results for CHIKV. We did not detect infections by ZIKV or DENV serotypes 3 and 4 in the analyzed samples.

The mean age of the monoinfected patient groups was 38.02 years (SD ± 20.09) (CHIKV); 37.93 years (SD ± 17.39) (DENV1) and 33.00 years (SD ± 11.80) (DENV2). Coinfected patients had a mean age of 31.20 years (SD ± 12.40). Of the total patients involved in this study, 67 (65.69%) were women and 35 (34.31%) were men. The mean cycling threshold (Ct) of the monoinfected by CHIKV was 32.98 (SD ± 4.44), by DENV1 was 36.67(SD ± 2.57) and by DENV2 was 35.50 (SD ± 3.85). For the coinfecteds, it was 31.57 (SD ± 5.46) (Table 2).

Discussion

In this study, we molecularly characterized DENV and CHIKV infections and also identified patients coinfected with these viruses in the state of Tocantins. The frequency of monoinfected with DENV and CHIKV was high and coinfections between them were observed in five patients. There is still little information available on co-circulation and coinfection of arbovirus in Brazil, especially in the northern region of the country. Thus, our study had the objective of contribute to this field of knowledge helping to subsidize public policies that ensure improvements in the diagnosis of arboviruses in Brazil and in world.

The coexistence of DENV and CHIKV has been documented in many regions of the world. In an outbreak of feverish illness that occurred in Gabon in 2007, with 20,000 suspected cases, DENV2 and CHIKV infections were identified in 321 patients, 8 of them being diagnosed with coinfection between these viruses [19]. In India, CHIKV reemerged in late 2005 overlapping the endemic areas of DENV. Thus, the frequency of concomitant infection with both viruses has increased notably in the country [20]. A cross-sectional study conducted through a 2008–2018 data review in Colombia showed that DENV is endemic in the country and that CHIKV emerged in 2014, circulating simultaneously with DENV [21].

In Brazil, since 2014, the co-circulation and coinfection of different arboviruses of importance in public health have been reported. A recent study screened the cases of DENV, CHIKV and ZIKV in the state of Minas Gerais and a total of 11 patients were diagnosed with coinfection, five coinfections involving DENV and CHIKV [22]. A work recently realized in Salvador in state of the Bahia investigated clinical and epidemiological characteristics of arboviral infections during the introduction and spread of CHIKV and ZIKV throughout northeastern Brazil. From total of 246 samples with acute arboviral infection analyzed, 23 were characterized with coinfection and 13 coinfections between DENV and CHIKV [23]. It reinforces the idea of co-distribution and independent transmission of these arboviruses in the country.

According to Malavige et al. [24], the initial clinical manifestation in DENV infection results in high fever accompanied by myalgia, arthralgia, exanthema, headaches and retro-orbital pain. In case of mild monoinfection these symptoms subside within 10–15 days, but may worsen in cases of severe Dengue. In accordance with Cunha and Trinta [25], in a mild CHIKV monoinfection, clinical manifestations are similar to those of a DENV infection, but the most distinguishing feature of Chikungunya infection is severe polyarthralgia, which persists for 3–12 months and may become chronic and debilitating. Thus, the clinical findings of patients characterized with monoinfection in our study corroborate with these studies previously mentioned. From DENV monoinfected group, we observed that the symptoms fever, headache, exanthema, myalgia, arthralgia, body pain, and retro-orbital pain were highly prevalent in individuals infected with both serotypes (1 and 2). In the group of CHIKV monoinfected patients the symptoms fever (89.3%), headache (78.7%), arthralgia (91.4%) and generalized body pain (93.6%) were more frequent. Edwards et al. [26] studying co-infections with Chikungunya and Dengue virus in Guatemala in 2015 showed that most CHIKV monoinfected patients had fever (90%), arthralgia (93%) and headache (85%), according to our findings clinicians.

Another important finding in our work was the detection of people with double infection. Some studies show that dual infection with Dengue and Chikungunya viruses is associated with more severe clinical diseases and therefore the pathogenesis of this association needs to be better understood [27–29]. However, we did not observe severe symptoms in the five patients detected with DENV/CHIKV coinfection. Fever, arthralgia, rash, nausea, conjunctivitis, retro-orbital pain, headache, leukopenia, myalgia and vomiting were the common clinical aspects of these people corroborating with a comparative study of clinical features between cases of monoinfection and coinfection with DENV and CHIKV in India, where the authors point out that these clinical features are common in both types of infection [30].

It is worth mentioning also that we found leukopenia only in patients with double infection. Singha et al. [31], analyzing the clinical profile of Dengue and Chikungunya coinfection, showed 13 coinfected patients. Of these, 7 presented with leukopenia. Consistent with this, another study conducted by Kularatne et al. [32] reported that leukopenia was a clinical feature present in monoinfected and coinfected patients by DENV and CHIKV. This indicates that leukopenia may be present in people infected simultaneously by both viruses and also in patients with a single infection. However, in our study we did not observe this clinical aspect in monoinfected groups.

This combination of concomitant circulating of arboviruses in Brazil presents a major challenge in national public health regarding case confirmation. The patients of this study are adults living in different cities of the state of Tocantins. However, there are some limitations in our work due the low number of clinical samples that we received. As it was a retrospective study we could not get complete medical information. In addition, serological analyses were not performed.

This work reinforced that there is simultaneous circulation of different arboviruses in Tocantins; however, more studies of molecular level need to be performed for confirm possible circulation of other arboviruses (not DENV, CHIKV and ZIKV) and also detection of coinfection among them in the state. In 2017, some specific cases of infection by yellow fever virus in humans were documented. Tocantins is known as endemic for this virus for approximately 30 years; however, studies that molecularly

characterize this reemerging virus may help to understand its reintroduction. In addition, recent reports have shown the presence of mayaro virus in the states of Mato Grosso, Goias and Rio de Janeiro, what come evidencing its spread throughout the country. The next step in our study will be to analyze molecularly in samples that were negative for CHIKV; possible infection by mayaro virus.

Conclusion

The molecular tests performed in this study were effective in confirming the presence of DENV, CHIKV and ZIKV and also made it possible the detection of simultaneous infections between DENV and CHIKV in clinical samples from the state of Tocantins. Thus, our findings suggest that clinical and epidemiological criteria are not sufficient to differentiate arbovirus infections, reinforcing the need a molecular diagnosis.

Authors' contribution

RSSM, RLSD and GLS were responsible for the planning and execution of the experiments performed; RSSM also interpreted the data and drafted the manuscript; JH performed the statistical analyses and participated in the writing of the manuscript; MART, RB and FAPM were responsible for processing and transport of the samples to the Laboratory of Retrovirology; RSD is the head of the Laboratory of Retrovirology where we carry out this study; ECS was involved in the interpretation of the data and SVK was involved in the planning of the experiments performed, discussion of the data and helped in writing of the manuscript.

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Competing interests

None declared.

Ethical approval

Not required.

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