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Original article

Zika virus RNA detection in blood donors in São Paulo, Brazil

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ABSTRACT

Introduction: The Zika Virus (ZIKV) is a single-stranded RNA genome virus, belonging to the family Flaviviridae, genus Flavivirus. Outbreaks around the world have demonstrated that the presence of asymptomatic viremic blood donors provides an increase in the risk of transfusion transmission (TT) and nucleic acid test (NAT) screening has been proposed to ensure the blood safety. This study implemented an "in-house" method to detect ZIKV RNA in blood sample donations.

Methods: Primary plasma tubes are submitted to nucleic acid extraction on an automated platform. After extraction, the NAT set-up is performed in the robotic pipettor, in which an amplification mixture containing primers and probes for ZIKV and Polio vaccine virus (PV) are added in duplex as an internal control. The real-time polymerase chain reaction is then performed in a thermocycler, using the protocol established by the supplier.

Results: From May 2016 to May 2018, 3,369 samples were collected from 3,221 blood donors (confidence coefficient 95%), of which 31 were considered false positive (0.92%), as they did not confirm initial reactivity when repeated in duplicates and 14 (0.42%) had their results invalid due to repeat failure in the internal control, 4 (0.12%), due to insufficient sample volume and 2 (0.05%), due to automatic pipettor failures. No Zika RNA reactive sample was identified.

Conclusion: The test showed feasible to be incorporated into the blood screening routine. Our data do not indicate the need to screen for ZIKV RNA in São Paulo during the evaluated period. However, a generic NAT system covering a group of flaviviruses which are circulating in the region, such as DENV and YFV, among others, could be a useful tool.

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Introduction

Zika Virus (ZIKV) is a single-stranded RNA genome virus, positive-sense, non-segmented, belonging to the family

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Flaviviridae, genus *Flavivirus*, which also includes Dengue virus (DENV) and West Nile virus (WNV). Since it was first isolated in 1947 in samples of rhesus monkeys in the Zika Forest, Uganda, during studies on yellow fever,[1] ZIKV infections of the ancestral African lineage were limited to enzootic circulation between non-human primates and sylvatic aedes mosquitoes.[2] As ZIKV migrated to Asia, the Asian lineage emerged, capable of being transmitted by the human-adapted *aedes* mosquitoes. However, ZIKV infections in humans were sporadic, with fewer than 20 cases reported before 2007[3]. Thenceforth, outbreaks were reported after this period in Yap Islands, Micronesia in 2007,[3,4] French Polynesia in 2013 and 2014,[5] Brazil in 2015, followed by the pandemic spread of the virus between 2016 and 2017, demonstrating an important change in the epidemiological behavior of the virus.

The clinical presentation of the disease varies, with 40% to 80% of asymptomatic and oligosymptomatic self-limited cases, and may manifest with mild maculopapular exanthema, fever, arthralgia and conjunctivitis,[6] up to severe forms of neurological impairment, such as the Guillain-Barre Syndrome[7,8] and microcephaly in fetuses and newborns with a history of maternal infection during pregnancy.[9,10] The clinical association between ZIKV and birth defects led to the recognition by the World Health Organization (WHO) of a global public health emergency in February 2016.[11]

The main form of transmission in humans occurs through mosquito bites, especially of the Aedes species (e.g., *Aedes aegypti*), however, non-vectorial transmission by sexual relations is also described,[12,13] in addition to intrauterine and perinatal contamination,[14] organ transplantation and transfusion of viremic blood components. A high prevalence of asymptomatic viremic donors at the time of blood donation has been reported during outbreaks in Martinique,[15] French Polynesia[16] and Brazil[17] and the description of transfusion-transmitted Zika virus (TT-ZIKV) in Brazil[18,19] led to the implementation of various preventive measures aiming at transfusion safety in endemic and non-endemic regions,[20,21] including donor self-reporting of ZIKV symptoms after donations, quarantine of blood components, pathogen inactivation of plasma and platelet blood products, as well as donor testing for ZIKV RNA.

The primary objective of this study was to develop a real-time polymerase chain reaction (RT- PCR) for the detection of ZIKV RNA at the blood bank at the Hospital Israelita Albert Einstein i n São Paulo, Brazil and apply it to routine screening of blood donors. Secondarily, we evaluated the soroprevalence by testing for ZIKV IgG and considering potential cross-reactivity among other flaviviruses for DENV IgG and for ZIKV IgM, in a complementary manner.

Methods

The blood bank at the Hospital Israelita Albert Einstein (HIAE), located in the city of São Paulo, Brazil, conducts an average collection of 1,100 blood bags and transfuses 950 units every month, of which 63.1% are RBC concentrates, 28.4% are platelet concentrates, 8% fresh frozen plasma and 0.5% cryoprecipitate, especially in intensive care units and oncohematology clinics, as well as bone marrow transplantation. Between May 2016 and May 2018, all candidates for blood donation who attended the blood bank of the HIAE, were submitted to a clinical questionnaire and a medical examination conducted by nurses, which included the family and health histories and an account of recent travel, according to Brazilian law for the pre-donation interview. Candidates with a history of Zika diagnosis in the past 30 days or sexual contact with individuals who had a clinical or laboratory diagnosis of ZIKV in the past 90 days, were deferred from blood donation for 30 days after complete clinical recovery or after sexual contact, respectively. A history of travel to regions with a high incidence of ZIKV infection also led to deferral for a period of 30 days. Regions with a cumulative incidence above 100 cases/100,000 inhabitants in the last 12 months were considered endemic areas in Brazil, as published in the Epidemiological Bulletin of the Health Surveillance Secretariat/Ministry of Health, and disclosed on the ANVISA website. For regions outside Brazil, the source of information for travel-related deferrals was the Centers of Disease Control and Prevention (CDC) website . Consultations were held weekly.

Volunteer blood donors who met these criteria were invited to participate in the trial and, prior to the sampling, donors signed the informed consent form. The study was approved by the Ethics Committee of the Hospital Israelita Albert Einstein (CAAE: 62612716.3.0000.0071).

Five milliliters of blood were collected in sterile EDTA tubes (BD, Vacutainer PPT) and samples were identified with a unique donation number, according to the International Society of Blood Transfusion (ISBT) standard for the HIAE Blood Bank Service, ensuring confidentiality of the donor's identity. Plasma was separated by centrifugation and tested individually.

The viral RNA was extracted from 1 mL of plasma in the QIAsymphony (Qiagen, Germany) equipment, using the DSP Virus/Pathogen MidiKit, following the CELLFREE1000_V7_DSP protocol, according to the manufacturer's instructions. Before extraction, samples were spiked with the Sabin vaccine Polio Virus (PV) diluted 1/100 in purified water, as an internal control for the whole process. The positive control of the reaction was a 1:100,000 dilution of a ZIKV culture supernatant from the Asian lineage, derived from a donor positive plasma.[18] Negative controls were plasma samples from donors previously tested for ZIKV.

The one-step RT-PCR was performed with the QuantiNovaRT-PCR kit (Qiagen) with a pair of primers and a probe specific for the ZIKV, previously described in the literature,[22] and a pair of primers and a probe for the Poliovirus. The RT-PCR was performed in a duplex reaction.

The amplification was performed in a final volume of 20 μL containing 8.8μL of extracted RNA, ZIKV and poliovirus primers in a concentration of 0.5μM, ZIKV and poliovirus probes in a concentration of 0.25μM, 1x Quantinova Probe RT-PCR Mix. The set-up was achieved with the QIAgility automatic pipettor (Qiagen). The real-time PCR was run on the Rotor-Gene equipment (Qiagen) with the following cycling profile: an initial incubation of 10 minutes at 45°C then 5 minutes at 95°C, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. Analyses of the amplification curves were performed by the equipment software, with a threshold setting between 0.070 to 1,000 for the ZIKV curve, and 0.040, for the PV curve. Samples that

showed amplification above the threshold were considered positive.

The analytical sensitivity was determined by the Probit Analysis, using the statistical software, IBM Corp SPSS version 2.0 and expressed as a 95% hit rate. A quantified ZIKV RNA standard, provided by the World Health Organization (WHO) and the Paul Ehrlich Institute, containing 7.5-8.5 log10 copies/ml was diluted with donor negative plasma to reach final concentrations of 500IU/mL, 250UI/mL, 100UI/mL, 50UI/mL, 5UI/mL and 0.5UI/mL. For each concentration, 2 extractions and 6 amplifications were performed on 4 different days, totaling 24 replicates. The obtained analytical sensitivity was 153 IU/mL. The specificity of the test was determined through the qualitative analysis of standard samples, provided by WHO and the Paul Ehrlich Institute, containing the different serotypes of the dengue virus: DENV-1, DENV-2, DENV-3 and DENV-4.

Samples found initially positive for ZIKV RNA were also tested with the Transcription Mediated Amplification (TMA), using the Aptima® Zika virus assay (Hologic), at the Vitalant Research Institute (San Francisco, USA).

Donor seroprevalence was assessed with 140 donations collected, due to the seasonal feature of the infection, between May and July 2016 and between January and July 2017 and were submitted to the ZIKV IgG (Euroimmun, Germany) testing.

All the ZIKV IgG positive samples were tested for DENV IgG and ZIKV IgM.

The DENV IgG test was performed using the Panbio® Dengue IgG Indirect ELISA (Abbott) at the Clinical Laboratory of HIAE. The ZIKV IgM was performed using the ZIKV Detect™ 2.0 IgM Capture test (Inbios) at the Vitalant Research Institute (San Francisco, USA). The purpose of performing these analyses was to exclude the ZIKV IgG false-positivity due to a potential cross-reaction between ZIKV IgG and DENV IgG.[23,24]

The ZIKV IgM test was performed using three different antigens: ZIKV ready-to-use recombinant antigens (RTU), cross-reaction control antigen (CCA), comprising dengue virus recombinant (DENV) and West Nile virus (WNV),[25,26]and a normal antigen control (NCA). Results were obtained by reading the optical density at 450 nanometers (DO450) of the samples containing the three different types of antigens. The result of the ZIKV IgM ISR (Immune Status Ratio) was calculated by the optical density ratio found in the samples containing the ZIKV recombinant antigen (ZIKV AG DO45) and the cross-reaction control antigen (CCA DO45). The CCA / NCA measurement was calculated from the optical density ratio found in the samples containing the cross-reaction control antigen (CCA DO45) and the normal antigen control (NCA DO450). The final interpretation of the ZIKV IgM Detect™2.0 IgM Capture test was based on ZIKV IgM ISR and ZIKV IgM CCA/NCA results, for which, if ZIKV IgM ISR ≥ 1.70, the sample was considered Presumptive ZIKV Positive, if ZIKV ISR <1.70 and CCA / NCA ≥ 5.00, the sample was considered Presumptive Other Flavivirus and if ZIKV ISR < 1.70 and CCA/NCA < 5.00, the sample was considered ZIKV negative.

Results

A total of 3,369 ZIKV RNA samples were collected from 3,221 blood donors (confidence coefficient, 95%; supposed ZIKV RNA prevalence, 5%). Of the 3,369 samples tested, 31 were considered false positive (0.92%), as they did not confirm initial reactivity when repeated in duplicates and 14 (0.42%) had their results invalid due to repeat failure in the internal control, 4 (0.12%), due to insufficient sample volume and 2 (0.05%), due to automatic pipettor failures. The 31 initially reactive samples, subsequently considered false positives, were also tested with the TMA Aptima® Zika virus assay (Hologic) and were confirmed as negative. No ZIKV RNA positive sample was detected.

In the serology, the Zika seroprevalence was estimated by evaluating 140 donations, representing 4.15% of the cases. Seven samples presented reactivity by the Zika IgG assay. Among these seven ZIKV IgG positive samples, 6 were found DENV IgG positive. The 7 ZIKV IgG positive samples were also subjected to the ZIKV IgM Detect™2.0 IgM Capture test and 4 (2.85%) were classified as “PRESUMPTIVE ZIKV POSITIVE”, according to the manufacturer's instructions.

Table 1 demonstrates the results obtained for ZIKV IgG, DENV IgG, ZIKV IgM ISR, ZIKV IgM (CCA/NCA) and the final interpretation of ZIKV IgM results.

Thus, the adjusted ZIKV seroprevalence among our blood donor population is estimated at 2.85%.

Discussion

Historically, arboviruses (arthropod-borne viruses) are a challenge to public health because of their power to cause epidemics on a global scale, affecting millions of people, high morbidity rates, such as those evidenced by the severe neurological manifestations of the West Nile virus neuroinvasive disease and high mortality rates from dengue hemorrhagic fever and dengue shock syndrome. Unfortunately, there is a trend towards new epidemics caused by both newly discovered or re-emerging arboviruses due to human social

Table 1 – Serology results in 7 Zika IgG+ donations.

Sample ID	ZIKV RNA	ZIKV IgG status	DENV IgG status	ZIKV IgM (ISR)	ZIKV IgM (CCA/NCA)	ZIKV IgM Final interpretation
304408	NEGATIVE	POSITIVE	NEGATIVE	1.086	1.208	NEGATIVE
303316	NEGATIVE	POSITIVE	POSITIVE	17.176	1.085	PRESUMPTIVE ZIKV POSITIVE
312479	NEGATIVE	POSITIVE	POSITIVE	7.271	1.157	PRESUMPTIVE ZIKV POSITIVE
312935	NEGATIVE	POSITIVE	POSITIVE	0.202	7.915	PRESUMPTIVE OTHER FLAVIVIRUS
313154	NEGATIVE	POSITIVE	POSITIVE	3.422	1.071	PRESUMPTIVE ZIKA POSITIVE
320658	NEGATIVE	POSITIVE	POSITIVE	2.114	1.667	PRESUMPTIVE ZIKA POSITIVE
320662	NEGATIVE	POSITIVE	POSITIVE	0.833	1.333	NEGATIVE

behavioral changes, such as globalization and increased intercontinental travel, demographic changes, including migration of populations to areas with transmission by other hosts and vectors, and urbanization,[27,28] in addition to global warming, which favors vector expansion.

During epidemics, the high rate of asymptomatic viral donors has generated an alert in blood bank services as to the possibility of transfusion transmission and the need to implement effective prevention measures to ensure transfusion safety.[29] In this scenario, the first major response of the transfusion community to an arboviral threat occurred with the unquestionable evidence of the aggressiveness of WNV-TT in the United States (US) in 2002,[30] followed by the fast implementation of NAT for the screening of blood donations in the US in 2003,[31] and further in other European countries.[32,33] A different approach was adopted during the chikungunya virus (CHKV) outbreaks in 2005 - 2007 on La Reunión Island and when blood donation was discontinued as a precautionary measure in the areas involved[34] and red blood and plasma components were supplied by the Établissement Français du Sangue, the French National Transfusion Service. Because of the short shelf-life (5 days) of platelets, pathogen inactivation of apheresis platelets was implemented.[35,36]

Facing the challenging epidemic scenario of the ZIKV in Brazil in 2016 and the absence of available commercial tests for ZIKV RNA in blood donors, as well as to maintain the transfusion safety, we conducted a study to evaluate the prevalence of viremia in donors by an “in-house” real-time polymerase chain reaction (RT-PCR) for the detection of ZIKV RNA in individual samples. The similar strategy of using NAT for ZIKV in individual samples was adopted in other outbreaks, such as those in Martinique (2016),[15] Porto Rico (2016) and the whole US territory in face of the advancing epidemic in the country.[20,21,37] Our study demonstrated a low prevalence of ZIKV viremia in blood donors during the epidemic period registered in Brazil in 2016. These results differ from those previously described in French Polynesia and Ribeirão Preto, a city located in northeastern São Paulo State, where the viremic rates found were both of approximately 3%,[16,17] but are similar to the occurrences observed in the midwestern,[38] northeastern[39] and southern[40] Brazilian regions, suggesting different geographic distribution of the virus.

When we assessed official data on the number of autochthonous ZIKV cases in São Paulo City, provided by the Epidemiological Surveillance Center, only thirteen cases were confirmed in 2016, 3, in 2017 and zero, in 2018, for a total population of 11 million inhabitants. Eight of the thirteen confirmed cases in 2016 took place between January and April, which was prior to starting the data collection for this study. These data suggest that the 0% viremia prevalence rate reflects the small number of cases of ZIKV infection in São Paulo City during the years 2016, 2017 and 2018.

Additionally, we evaluated the seroprevalence rate of our donors and obtained a rate of 2.85% (4/140) of presumptive ZIKV. The ZIKV IgG still remains as the test of choice to assess past exposure and the objective of performing it was to provide supplementary surveillance data for the studied population due to its long stay in circulation. It is especially important to consider the presence of cross-reaction with other flaviviruses and, therefore, its distinction is necessary through complementary tests, such as those performed in our study. When we compare our results with the literature, we see that in regions without active transmission, the prevalence of anti-ZIKV IgG in donors ranges from 0.001 to 0.003%,[37,41] but in areas with active circulation, such as the African continent, this number can reach 4.89%.[42]

Some issues remain unknown in the history of ZIKV transfusion, such as the real risk of transmission and the clinical impact of ZIKV transfusion transmission (TT-ZIKV) to blood component recipients. Mathematical models have evaluated the transfusion risk of other arboviruses, such as WNV, DENV and CHKV, through formulas that correlate the prevalence in asymptomatic viral donors and the duration of the viremia period.[43] In these models, it is assumed that all viremic donations are capable of causing infection. For WNV, the first model was proposed in 1999 with an estimated maximum risk of 2.7 and the mean risk of 1.8 for every 10,000 donations, respectively,[44] and updated in 2002 to a medium risk of 2.12 to 4.76 and maximum risk of 4.32 to 10.46 for every 10,000 donations.[45] For ZIKV, this data is difficult to measure due to the short period of viremia and rapid reduction in viral load after the onset of symptoms. Magnus and collaborators demonstrated the existence of a residual risk of TT from ZIKV in the region of Campinas, located in the countryside of São Paulo State, where the first probable case of TT was described in 2015 during a period of low circulation. In their study, the prevalence of viremia in 2,000 blood donors was 0.16% (3/2,000).[46]

The clinical impact of ZIKV transfusion transmission on blood component recipients has not yet been thoroughly evaluated. A retrospective study performed in French Polynesia[47] followed 12 blood component recipients of ZIKV viremic units, in whom no clinical manifestations of infection were evidenced, similar to the data found for the two cases of TT −ZIKV described in Brazil.[18,19]

Although the clinical impact on recipients with TT ZIKV is uncertain, the precautionary principle should be adopted by blood bank services, in which transfusion risks mitigation measures should be instituted as early as possible in epidemic situations, especially for susceptible populations. Therefore, considering the severity of possible neurological manifestations of ZIKV in fetuses and newborns, the adoption of transfusion protocols with indications for the use of ZIKV RNA negative blood components for pregnant women is justified, as recommended by the WHO in 2016.[11]

The screening for ZIKV RNA in asymptomatic donors has been shown to be feasible and capable of providing important information, especially in susceptible populations with active transmission of infection. Our data do not indicate the need for screening for ZIKV RNA in São Paulo City during the evaluated period. However, a generic NAT system covering a group of flaviviruses which are circulating in the region, such as ZIKV, DENV and YFV, among others, could be a useful tool.

Conflicts of interest

The authors declare no conflicts of interest.

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