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Research article

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Circulation of DENV2 and DENV4 in *Aedes aegypti* (Diptera: Culicidae) mosquitoes from Praia, Santiago Island, Cabo Verde

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Abstract

Arthropod-borne viruses, such as Dengue (DENV), Chikungunya (CHIKV), and Zika (ZIKV), pose a challenge to public health, due to their worldwide distribution and large-scale outbreaks. Dengue fever is currently one of the most important diseases and it is caused by four serotypes of DENV and is mainly transmitted by the mosquito *Aedes aegypti*. It is estimated that 50–100 million cases are reported every year worldwide. More recently, CHIKV and ZIKV, which are also transmitted by *Ae. aegypti*, have caused epidemics in countries in the Caribbean region, the Pacific region, and Americas. Cabo Verde faced its first dengue outbreak in 2009, with more than 21,000 reported cases and four registered deaths. The epidemic was caused by DENV-3 transmitted by *Ae. aegypti* mosquitoes. In addition, the country faced a Zika outbreak with more than 7,500 notified cases from October 2015 to May 2016. In the present study, we conducted a survey in mosquito samples to detect arboviruses circulating in the local vector population. Collections were performed from November 2014 to January 2015, in the City of Praia, the capital of Cabo Verde, using aspirators and BG-sentinel traps. Samples were examined by multiplex Reverse Transcription-polymerase chain reaction. A total of 161 *Ae. aegypti* adult females were analyzed (34 pools) and from these samples, eight pools were found positive for DENV-2 and DENV-4. Our results revealed a very high natural infection rate in the vector population and showed two different serotypes co-circulating in the island that differ from the one detected in the 2009 outbreak in Cabo Verde.

Key words: *Aedes aegypti*, dengue, diagnostics, arboviral transmission

In recent decades, the world has faced a drastic emergence and re-emergence of some arboviruses, particularly dengue (DENV), chikungunya (CHIKV), and zika (ZIKV) viruses. These three viruses are transmitted mainly by *Aedes aegypti*, a mosquito species historically targeted for control due to its great importance in the transmission of urban yellow fever (YF). Fortunately, a vaccine has been available for YF virus since 1951 (Monath 2001), which helps to diminish the risk of YF, although outbreaks have recently been reported (Lancet 2016, WHO 2016a). The first vaccine for DENV was developed by the French company Sanofi and has completed a phase III trial in Mexico (Schwartz et al. 2015). A second vaccine developed by Instituto Butantan, a Brazilian Institution, is in the last stage of clinical trials (Precioso et al. 2015). However, for CHIKV and ZIKV, no vaccines are yet available. Due to the lack of available vaccines, the management of these diseases (dengue, chikungunya, and zika) is mainly achieved by vector control through source reduction and the use of chemical insecticides. These three diseases together cause more than 100 million cases a year. It is worth noting that in addition to the severe dengue cases, which cause several deaths, zika has recently been linked to serious manifestations such as Guilain-Barré syndrome (Oehler et al. 2014) and congenital zika virus syndrome cases. In Brazil, until September 2016, 9,514 cases of microcephaly and/or neurological alteration leading to death had been associated to ZIKV infection. A total of 1,949 cases were confirmed, while 3,030 cases are

still under evaluation (MS-BRASIL 2016, Moron et al. 2016, Santos and Goldenberg 2016; Schuler-Faccini et al. 2016). The rapid spread of ZIKV in South and Central America occurred in localities previously exposed to DENV. The serological interaction between DENV and ZIKV might explain the clinical complications, since DENV immunity induces a higher ZIKV replication rate (Dejnirattisai et al. 2016). Therefore, constant monitoring and surveillance systems must be implemented in areas where cases of arboviruses have been identified. The virological surveillance should support the health system in choosing the correct clinical management of the patients, and establishing the vector control procedures needed to prevent the spread of these diseases.

The co-circulation of DENV, CHIKV, and ZIKV has been reported in many tropical countries such as Brazil (Cardoso et al. 2015), French Polynesia (Aubry et al. 2015), New Caledonia (Dupont-Rouzeyrol et al. 2015), Mexico (Dzul-Manzanilla et al. 2015), and India (Chahar et al. 2009). The simultaneous presence of such arboviruses is a complicating factor for disease surveillance systems, as currently available serologic tests are unable to distinguish virus infections in patients who have had a previous flavivirus infection (Lanciotti et al. 2008). Therefore, more specific tests that rely on the detection of viral RNA by RT-polymerase chain reaction (PCR) are needed. However, these tests can only be performed within the viremia period for each infection (Patel et al., 2013).

In Cabo Verde (CV), an archipelago constituted by 10 islands located on the west coast of Africa, 540 km from Senegal, the first dengue cases were reported in 2009, when almost 22,000 cases were registered and four deaths reported (MS-CV 2010, 2016). The DENV-3 serotype was implicated in this outbreak, which was probably introduced from Senegal (Faye et al. 2014). After this epidemic, the country has registered a low number of dengue cases, probably due to the decline in the number of humans susceptible to this particular serotype. During the 2009 outbreak all islands, except Santo Antão and São Vicente Islands, reported autochtone dengue cases. Santiago, where the capital is located, notified most cases. In 2015, CV identified an outbreak of zika, in which more than 7,000 cases were reported from October 2015 to March 2016 (WHO 2016b). While CHIKV is endemic in Senegal, with multiple reported outbreaks (Diallo et al. 1999, 2012), and is also endemic in several other African countries, CV has never reported a case. It is important to highlight that *Ae. aegypti* mosquitoes from CV are susceptible to CHIKV and possess the potential to transmit this virus (Diagne et al. 2014).

In this context, entomological surveillance to detect the circulation of arboviruses in a given area could be a valuable tool to complement the diagnosis performed in the human population, especially in areas with the co-circulation of different arboviruses. It is important to highlight that no virological surveillance in human population were performed as a part of this study. We have used two RT-PCR systems to detect Flaviviruses [DENV and Yellow fever (YFV) viruses] and Alphaviruses [CHIKV, Mayaro (MAYV), Venezuelan Equine Encephalitis (VEEV), Estern Equine Encephalitis (EEEV), Western Equine Encephalitis (WEEV) and Aura (AURAV) viruses]) in mosquitoes collected from the field, based on Hasebe et al. (2002) and Bronzoni et al. (2005) protocols. Interestingly, CV is the only country in the African continent whose government publicly displays dengue cases (Brady et al. 2012), and therefore we could compare the circulation of viruses in the human and mosquito populations.

Materials and Methods

Study Area and Mosquito Collection

Ae. aegypti adult mosquitoes were collected in the City of Praia (14° 55′ 15″ N, 23° 30′ 30″ W), Santiago Island, CV, between November 2014 and January 2015 in three neighborhoods: Palmarejo, Tira Chapeu, and Fonton (Fig. 1). Mosquito collection was performed by mechanical aspiration (Nasci 1981) and BG-Sentinel traps (Biogents AG, Regensburg, Germany). After field collection, samples were taken to the laboratory at the Universidade Jean Piaget and identified using a biological identification key as described in Ribeiro et al. (1980). After species identification, specimens were sorted by sex and locality, and then stored by pool (up to six mosquitoes) in 1.5-ml tubes containing *RNAlater* (Qiagen, Hilden, Germany). Samples were kept at 4°C for 24 hours to allow *RNAlater* to impregnate the mosquito tissues, after which samples were transferred to a −20°C freezer. Mosquito samples were shipped to the Department of Entomology at the Instituto Aggeu Magalhães (IAM/FIOCRUZ-PE, Brazil).

RNA Extraction

RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA), according to manufacturer protocol and minor modifications as described in (Carvalho-Leandro et al. 2012). After extraction, all RNAs were treated with Turbo DNase (Invitrogen) and then stored at − 80 °C.

Molecular Detection for Arboviruses and Sequencing Reactions

All samples were initially assayed by RT-PCR for Chikungunya virus, as described by Hasebe et al. (2002), and with a minor modification of the addition of an rpL8 internal control (rpL8_fw: 5′ GCAAC CTGGAGGAGAAGACC3′ and rpL8_Rev: 5′ GGATTGTGGGCA ATGACGGA). This internal control was added to each reaction to evaluate RNA quality as described for DENV by Barbosa et al. (2016). Later, samples were assayed in a duplex RT-PCR (D-RT-PCR) as well as multi-nested-PCR (M-N-PCR) to identify arboviruses belonging to the *Flavivirus* (DENV and YFV) and *Alphavirus* (MAYV, VEEV, EEEV, WEEV and AURAV) genuses, as described by Bronzoni et al. (2005). RT-PCR products from positive samples were sequenced in both directions in an ABI Prism 3500xL Genetic Analyzer (Applied Biosystems, Foster City), using specific primers. Primers used for both RT-PCR and sequencing reactions are described in Supp Table 1 (online only). After sequencing, the evaluation of the quality of obtained sequences, assemble and editions were performed using the CodonCode Aligner software (version 3.7.1). After that, sequence identities were obtained by BLAST (Basic Local Alignment Search) (www.ncbi.nlm.nih.gov/BLAST) and latter were deposited in the GenBank database.

Minimum Infection Rate

Minimum infection rate (MIR) was calculated as the number of positive pools divided by the total number of mosquitoes tested multiplied by 1,000 (Chow et al. 1998).

Results

A total of 161 *Ae. aegypti* females were field-captured, from which one was caught by aspiration in Fonton, 19 in Tira Chapeu, and three in Palmarejo; the remaining 138 were caught by BG trap in Palmarejo. A total of 34 pools were analyzed by RT-PCR. Eight pools were positive for *Flavivirus* (all DENV): three pools were positive for DENV-2 (Fig. 2) and five pools positive for DENV-4. Most of positive pools (seven out of eight) came from the Palmarejo neighborhood, where the collection method was the BG-sentinel trap, and only one positive *pool* (DENV4)

Fig. 1. Collection sites (stars) of *Aedes aegypti*; adult females from Santiago Islands, Cabo Verde. Satellite map adapted from Google Earth. (A) Santiago Island. (B) Location sites in the city of Praia.

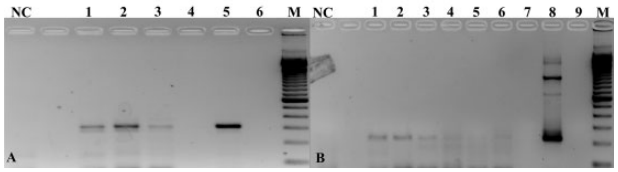

Fig. 2. DENV2 and DENV4 positive samples from *Aedes aegypti* mosquitoes collected in the city of Praia, Cabo Verde. (A) NC. Negative control; 1, 2 and 3. DENV2 positive samples (316 bp); 5. DENV2 positive control (DENV2 from cell culture) and M. 100 bp Ladder. (B) NC. Negative control; 1, 2, 3, 4, and 6. DENV4 positive samples (222 bp); 8. DENV4 positive control (DENV4 from cell culture) and M. 100 bp Ladder.

was collected by aspiration in Fonton. The MIR estimated in the present study was very high (49.69 per 1,000 females tested). No amplicons belonging to *Alphavirus* genus was detected. In addition, no virus isolation from positive samples were performed in the present study.

All positive samples were sequenced and five of them were confirmed as Dengue viruses with more than 98% identity each (Fig. 3). Sequences were deposited in GenBank, under the following accession numbers: KU577445 to KU577449. Three of the eight positive samples yielded poor quality sequences that could not be used to confirm their identity.

Discussion

In the present study, we have performed an arbovirus screening in *Ae. aegypti* samples from the city of Praia, CV, from November 2014 to January 2015. Two DENV serotypes (DENV-2 and DENV-4) were identified in eight positive pools of mosquitoes. Concerning virus surveillance in human population, it showed that DENV-3 was identified in a small proportion of human samples collected during the epidemic in 2009/2010. Since that time, no vector surveillance was performed in field-caught adult females. To our knowledge, vector surveillance was performed only in 2015 in mosquitoes obtained from the eggs, but no samples were positive (Moura et al. 2015). This study presents the first report of DENV-2 and DENV-4 circulation in the archipelago of CV.

The occurrence of 174 DHF cases and 4 deaths during the 2009/2010 epidemic raised the suspicion that these people may have had previous contact with other DENV serotypes, or were co-infected with another arbovirus such as CHIKV, which can frequently cause complications (Furuya-Kanamori et al., 2016). Nevertheless, in a study conducted in 2013 for transovarial transmission of DENV serotypes in four localities of Santiago Island, authors were not able to find any positive result for the presence of DENV serotypes (Moura et al. 2015).

The vector competence of *Ae. aegypti* populations from CV, to all DENV serotypes, was evaluated by Moura et al. (2015). They

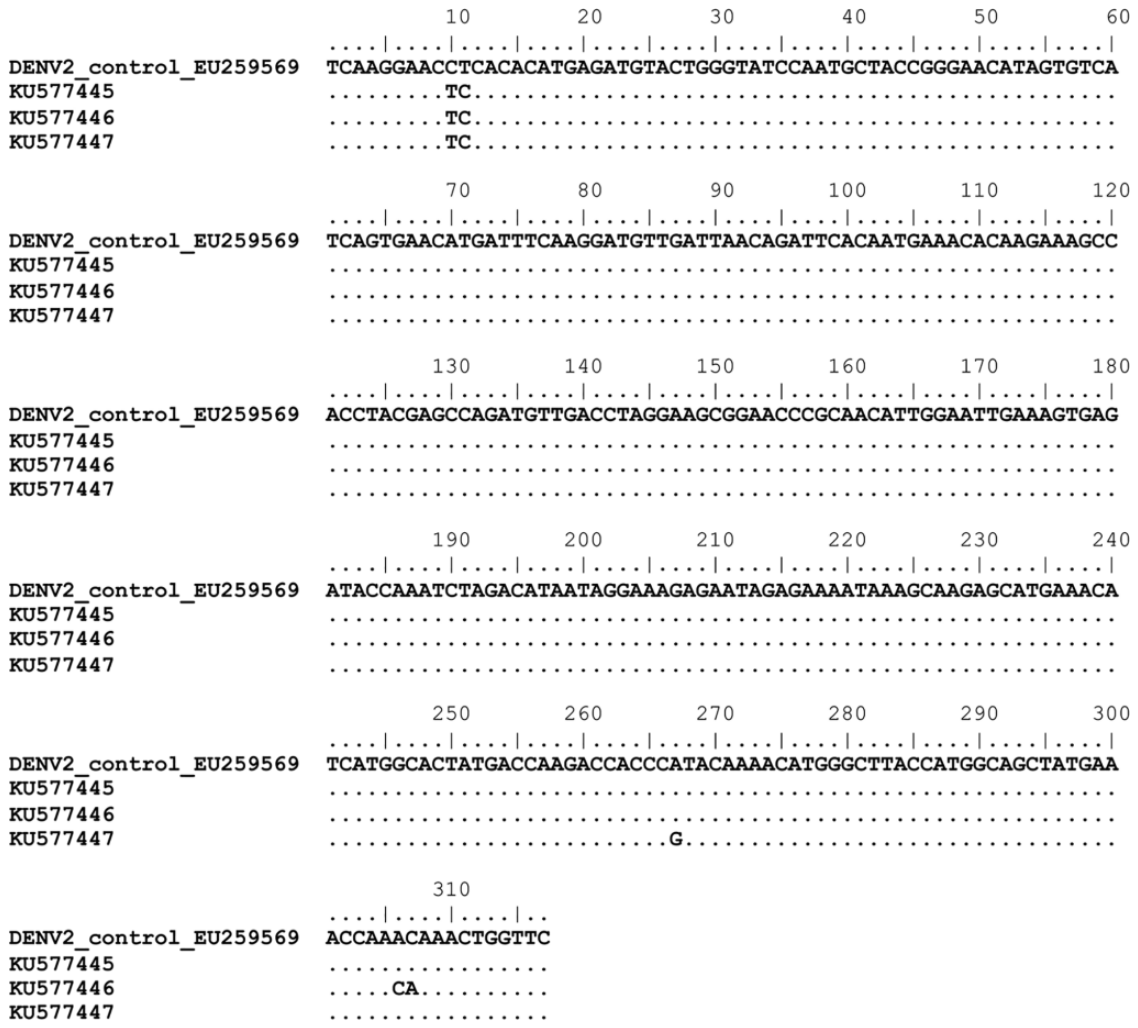

Fig. 3. Alignment of DENV-2 sequences from *Aedes aegypti* samples collected in the city of Praia, Cabo Verde.

showed that mosquitoes from CV were highly permissive to both infection and dissemination of DENV-2. However, the DENV-4 serotype did not spread to the head or salivary glands. Despite the lack of vector competence of *Ae. aegypti* populations from CV to DENV-4, we found field-caught mosquitoes infected with this serotype. This could be due to differences in the virus isolates used for vector competence assays, as it is well known that virus genotype *versus* mosquito genotype interactions can determine DENV transmission by *Ae. aegypti* (Lambrechts et al. 2009).

A second hypothesis is that those mosquito samples could represent newly fed mosquito females that fed on asymptomatic hosts, travelers coming from hyper endemic areas, such as Brazil, and are not necessarily capable of transmitting the virus. Duong et al. (2015) reported that asymptomatic people infected by Dengue are very common and can be infectious to mosquito.

Virological surveillance in mosquitoes has been used in studies elsewhere (Chow et al. 1998, Urdaneta et al. 2005, Guedes et al. 2010, Barbosa et al. 2016). The early detection of new DENV serotypes in mosquito samples can alert health authorities to a silent circulation of different DENV serotypes in human and/or vector populations in CV, as detected in Brazil (Guedes et al. 2010). It is also important to highlight recent studies showing the importance of previous Zika infections for Zika patients. Priyamvada et al (2016) have demonstrated that a previous DENV infection is able to induce cross-reactive ZIKV antibodies, boosting ZIKV infection in vitro. Similar results were obtained by Dejnirattisai et al. (2016) who concluded that dengue immunity may induce a higher ZIKV replication rate. Both results may explain the clinical severity of Zika infection observed in Brazil and other countries, where an overlap of ZIKV and other flaviviruses are found.

The recent increase in the number of tourists observed in CV, especially from countries experiencing Zika epidemics, may lead to a serious epidemiological situation on the islands. Vector surveillance can help public health programs to better direct their interventions to areas with a high risk of epidemics to prevent large-scale arboviruses outbreaks.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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