VECTOR-BORNE AND ZOONOTIC DISEASES
Volume XX, Number XX, 2019

DOI: 10.1089/vbz.2018.2411

Molecular Detection of Dengue Virus in Mosquitoes as an Early Indicator to Aid in the Prevention of Human Infection in Endemic Areas

André de Souza Leandro,[1] André da Silva Britto,[1] Jean Avemir Rios,[1] Sandro Roberto Galvão,[1] Rosinei Kafka,[1] Wagner Fabiano de Oliveira,[1] Orestes Fecci Neto,[1] Isaac Silva,[1] Robson Michael Delai,[2,3] Daniela Dib Gonçalves,[3] Walfrido Kühl Svoboda,[4] Açucena Veleh Rivas,[5] Renata Defante Lopes,[1] Flávia Julyana Pina Trench,[4] Wagner Antônio Chiba de Castro,[4] Alessandra Cristiane Sibim,[5] Luiz Fernando de Oliveira Ribas,[6] Fernando Rodrigues Gois,[7] Rafael Felipe da Costa Vieira,[7] and Alexander Welker Biondo[7,8]

Abstract

Human cases of dengue virus based on the National Dengue Control Plan were compared with the molecular detection of the dengue virus in trapped mosquitoes, verifying the prediction and efficacy potentials of vector control between the two methodologies in a city with three endemic frontiers. Molecular detection of dengue virus in trapped mosquitoes was significantly higher than in human cases ($p = 0.0435$). Thus, molecular detection could be used as an early indicator to help prevent more human cases of dengue.

Keywords: *Aedes aegypti*, *Aedes albopictus*, dengue fever, prevention

Introduction

Despite mosquito control efforts, arboviruses have exhibited an increasing incidence and a continuous geographic spread (Weaver 2014), including in Brazil (Medeiros et al. 2018), United States (Fauci and Morens 2016), and Europe (Schaffner and Mathis 2014). The Brazilian Ministry of Health has established a National Dengue Control Plan based on the notification of suspicious cases (before confirmatory laboratorial results), which immediately triggers an on-field vector control with chemical adulticide (MS 2009).

The World Health Organization Global Vector Control Response has set a goal for 2017–2030 aimed at reducing arbovirus loads and threats through rapid detection and outbreak control, and consequently decreasing global case incidence (WHO, 2017). Historical approaches in the battle against arboviruses have included new strategies for disease control and monitoring based on mosquito trapping (WHO 1967, WHO 1985, WHO 2016, WHO, 2017). Foz do Iguaçu had ~250,000 habitants at the time of the survey and ranked as the nation's second largest border city and the third most popular Brazilian tourist destination, with ~700,000 inhabitants when combined with the border city populations of Paraguayan Ciudad Del Lest and Argentinian Puerto Iguazú. In such a strategic commercial and touristic scenario, along with intense daily human traffic, Foz do Iguaçu may pose an international threat to public health because of the incidence of dengue fever, with 39.44 positive cases per 100,000 habitants in 2018. This incidence is considered high even for an endemic area (Brazil, 2019).

The growing One Health movement has been addressing the ecosystem in a coordinated manner, interrelating human, animal, and environmental health (Fao et. al. 2008). In this scenario, an interdisciplinary approach may be required to better understand the spread, fluctuations, epidemics, and

[1]Zoonoses Surveillance Unit, Municipal Secretary of Health, Foz do Iguaçu, Paraná, Brazil.
[2]One Health Laboratory at the Three-Border Tropical Medicine Center, Itaiguapy Foundation, Institute of Teaching and Research, Foz do Iguaçu, Paraná, Brazil.
[3]Department of Preventive Veterinary Medicine and Public Health, Paranaense University, Umuarama, Paraná, Brazil.
[4]Latin-American Institute of Life Sciences and Nature, Federal University of Latin American Integration, Foz do Iguaçu, Paraná, Brazil.
[5]Latin-American Institute of Technology, Infrastructure and Territory, Federal University of Latin American Integration, Foz do Iguaçu, Paraná, Brazil.
[6]Itaipu Binational and Department of Clinic Medicine, Federal University of Paraná, Paraná, Brazil.
[7]Department of Veterinary Medicine, Federal University of Paraná, Paraná, Brazil.
[8]Department of Comparative Pathobiology Purdue University, West Lafayette, Indiana.

outbreaks of infectious pathogens (Woldehanna and Zimicki 2015), particularly vector-borne agents, such as arboviruses.

The Brazilian Ministry of Health has created a National Program of Dengue Control (NPDC), with the goal to maintaining *Aedes aegypti* populations below a standard level, which has been established based on surveys of immature forms and the absence of epidemics. On-field actions have included larval combat through household visits and removal of breeding water places, such as tires and vases.

Furthermore, an insecticide is used when removal of water sources is impossible, and insecticides are sprayed in highly populated areas for adult combat (MS 2009). Notification of suspicious cases, before laboratory confirmation, has been the basis for on-field vector control with chemical insecticides, following the guidelines of the Pan American and World Health Organizations (Maciel de Freitas et al. 2014).

Despite the availability of mosquito trapping procedures, to date, no study has compared the official Brazilian protocol, based on suspicious cases of dengue fever as provided by the National Dengue Control Program, with molecular detection of dengue virus in mosquitoes, as the basis for dengue vector control. Accordingly, the goal of this study was to compare the official Brazilian protocol of vector control based on suspicious dengue cases with molecular dengue virus detection in trapped mosquitoes as a predictor for vector control in Foz do Iguaçu, a dengue endemic city, enclosed within the national borders of Brazil, Argentina, and Paraguay.

Materials and Methods

Foz do Iguaçu city (25°32′49″S, 54°35′11″W), with ~250,000 habitants distributed >617.70 km^2 (238.5 miles^2), has been classified as the largest border city and the third largest tourist destination in Brazil (IBGE 2017). The city is enclosed within the southern Brazilian, northern Argentinian, and eastern Paraguayan borders (Fig. 1).

The region has a subtropical climate with an annual average temperature of 23.8°C and two distinctive seasons consisting of a humid and hot summer and dry and cold winter. The city was chosen because of its unique strategic geographic location and current endemic status as the second most affected city in Parana state with 6309 Dengue cases in 2016. Mosquito traps have been routinely used by the city Zoonoses Surveillance Unit for *A. aegypti* attraction and trapping, as previously described (Gomes et al. 2007, Codeço et al. 2015). The survey was conducted in March because it has historically been the peak season of dengue occurrence locally.

A total of 3476 Adultraps®, as previously described (Donatti and Gomes 2007), were distributed homogeneously throughout the urban area of the city from 6 to 10 March 2017 by the Zoonoses Surveillance Unit. The domicile where each trap was installed was chosen randomly in an area with 25 households of average, so each trap monitored an average of 25 households.

The Adultrap is a trap designed to capture *A. aegypti*. It has a spherical shape and dark color, consisting of one chamber for baiting, one chamber for the entrance of adult mosquitoes, and a third to trap the mosquitoes inside (Donatti and Gomes 2007). The mosquitoes were individually subjected to qPCR or in pools containing two to eight specimens, according to the number of mosquitoes caught per trap or group of traps.

RNA extraction from the pools was performed using the MagMAX™-96 viral RNA isolation kit, as previously reported (Thermo Fisher Scientific, 2013). There were 51 qPCR exams in the system with a commercial triplex vector screening kit for dengue, zika, and chikungunya viruses (QuantStudio 7 and TaqMan One Step Multiplex; Thermo Fisher Scientific, Waltham, MA) at the One Health Laboratory, Foz do Iguaçu.

Although both *A. aegypti* and *Aedes albopictus* have been implicated in dengue transmission, a previous study in another Brazilian endemic area showed significantly lower prevalence (76/1360; 5%) and a lower infection rate (5/24; 20.8%) of dengue virus from *A. albopictus* when compared with *A. aegypti* (Medeiros et al. 2018). In addition, dead mosquitoes were discharged as testing samples because of potential mosquito and consequently virus degradation leading to bias results.

After mosquito capture and identification, the live *A. aegypti* females were submitted to RNA extraction and tested to a real-time PCR (qPCR) system with a commercial triplex vector screening kit for dengue, zika, and chikungunya viruses (QuantStudio 7 and TaqMan One Step Multiplex; Thermo Fisher Scientific) at the One Health Laboratory, Foz do Iguaçu.

The statistical package R (R Core Team 2019) was used for the statistical analysis. Normality of data distribution (notified cases for each group) was determined by the Shapiro–Wilk test. Because data distribution was considered nonparametric, the Mann–Whitney test has been applied to determine significant differences between groups.

Results and Discussion

The city service personnel were able to inspect only 2606/3476 (74.97%) installed traps, primarily because of some households being closed at the time of inspection. Overall, 694 mosquitoes were found in 294/2606 (11.28%) traps, of which 484/694 (69.74%) mosquitoes from 238/294 (80.95%) traps were identified as *A. aegypti*. Of the remaining 210/694 (30.25%) mosquitoes, 198/694 (28.53%) were identified as *Culex* sp., of which 77 males were from 32 traps and 130 females were from 62 traps. In addition, 12/694 (1.72%) from 10/294 (3.40%) traps were identified as females of *A. albopictus*.

Although dengue virus has been previously detected in mosquitoes up to 28 days after death, virus was experimentally detected using an antibody rather than through molecular approach (Thenmozhi et al. 2000, 2005, Paramasivan et al. 2006, Srisuphanunt et al. 2007, Voge et al. 2013, Sylvestre et al. 2014, Shukla et al. 2017). Because the goal of this study was to test live mosquitoes to ensure sample quality, even assuming underestimation of viral detection, 381/484 (78.72%) dead mosquitoes were excluded from molecular tests and discarded.

Furthermore, dead mosquitoes could compromise real-time PCR analyses because of no reliable and fluctuating lapse of time after death (possibly longer than 28 days), under different climate and environmental conditions. In addition, real-time PCR may allow a faster and more efficient response (Warrilow et al. 2002, Liotta et al. 2005, Samuel and Tyagi 2006, Blow et al. 2008, Gurukumar et al. 2009) regarding detection of dengue, zika, and chikungunya variants, and a combination of tests may be used to increase reliable and concomitant detection (Mardekian and Roberts 2015, Gutiérrez-Bugallo et al. 2018, Glushakova et al. 2019).

Out of *A. aegypti* specimens, 484 mosquitoes were found in 238/2606 (11.28%) traps, 435 females and 49 males,

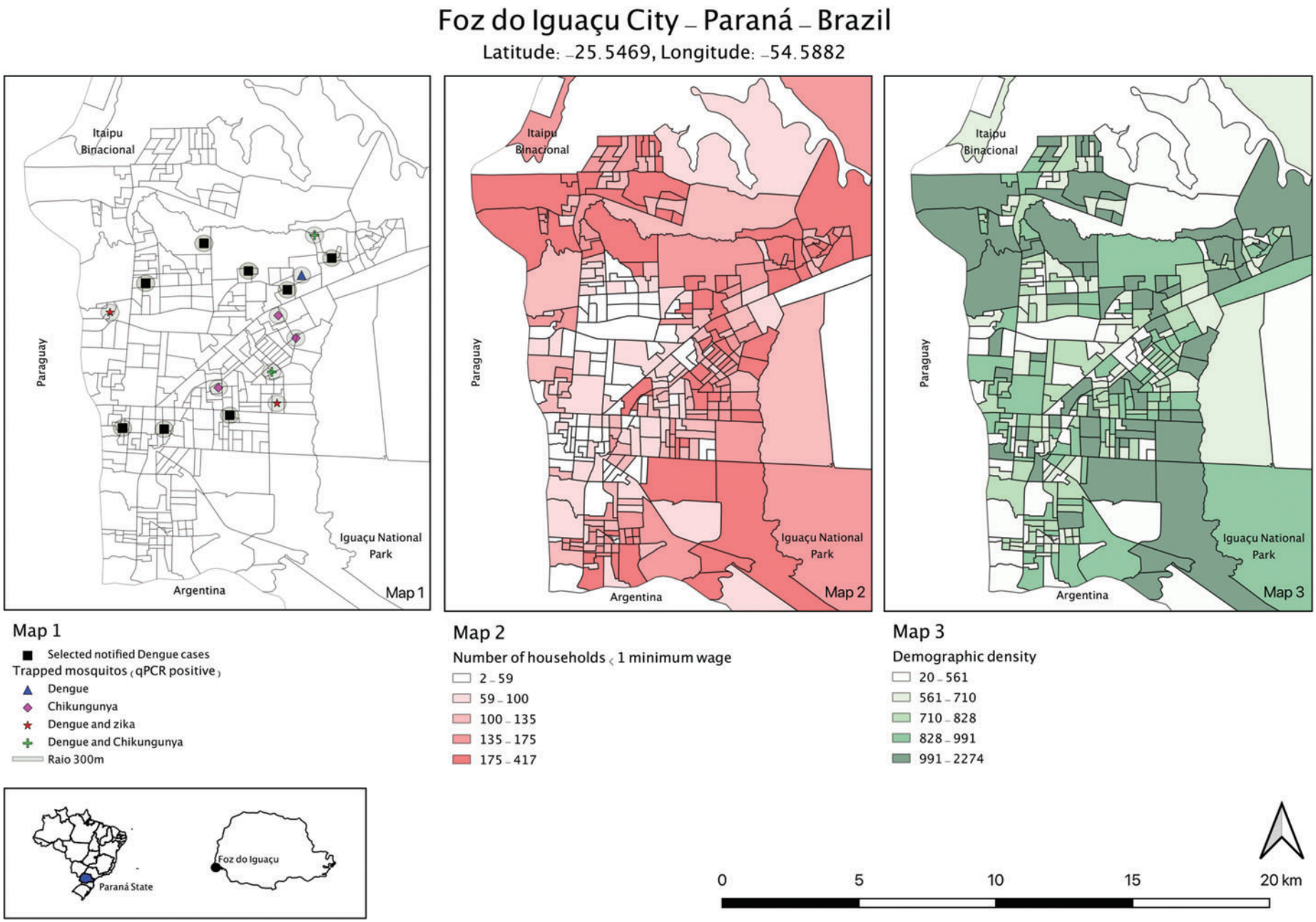

FIG. 1. Map indicating mosquito traps, HCGs, and sampling areas of HCG and PMG in the city of Foz do Iguaçu, southern Brazil, from March 6 to 10, 2017. HCG, human case group; PMG, positive mosquito group. Color images are available online.

varying from 1 to 13 mosquitoes per trap with an average of 1.04/trap. A total of 103/484 (21.28%) mosquitoes were found alive, of which 76 females were from 51 traps, varying from 1 to 8 mosquitoes per trap and an average of 1.49 live females/trap. A total of 49 *A. aegypti* male mosquitoes were found, of which 27 were alive and 22 were dead. After mosquito capture and identification, the 76 live *A. aegypti* females were mixed within each trap and submitted to a qPCR system (Table 1).

A total of 8/51 (15.69%) traps presented positive mosquitoes in the qPCR, of which 1/51 was positive for dengue, 3/51 for chikungunya, 2/51 for dengue and chikungunya, and 2/51 for dengue and zika (Fig. 2). The positive mosquito group (PMG) underwent an immediate on-field chemical vector control in a 300 m radius from the original location of each positive mosquito trap.

Simultaneous to mosquito trapping and testing, following the NPDC, 8/56 (14.28%) human cases were independently and randomly selected during the period and comprised the human case group (HCG). Immediate vector control was conducted in a 300 m radius from the patient household, presenting similar neighborhood patterns of population and income (Fig. 1).

No previous human case was reported surrounding the eight positive traps during the period. To avoid biased results, samples for the HCG were defined *a posteriori* based on the random occurrence of dengue virus in trapped mosquitoes, and no paired statistical methodology was used. All vector control activities were blindly performed by the Vector Control Section of the Zoonoses Surveillance Center, following the NPDC recognized by the Ministry of Health (MS 2002). Delimitation of sampling areas was performed by geoprocessing, using available commercial software (Quantum GIS and PostgreSQL). After the vector control actions, all human cases were recorded during seven epidemiological weeks (49 days) in both groups.

Table 1. Number of Notified Cases, Average Family Income, and Estimative of Inhabitants per Area, Comparing Human Case and Positive Mosquito Groups in the City of Foz do Iguaçu, Southern Brazil, 2017

			Income	*Habitants*
Group	*Block*	*Notified cases*	*(Minimal wage)*[a]	*(3.2 persons per household)*
Human case	1t	3	2.5	1312
	2t	3	2.3	2880
	3t	3	2.4	2950
	4t	3	2.7	1046
	5t	1	2.4	1325
	6t	3	3.2	2422
	7t	3	1.6	819
	8t	1	3.2	816
Positive mosquito	1v	0	2.5	3322
	2v	4	2.5	1194
	3v	2	1.7	2016
	4v	0	3.4	1450
	5v	0	2.2	1101
	6v	2	2.3	1094
	7v	0	2.3	1677
	8v	1	1.7	851

[a]The Brazilian minimal wage at the time was equal to US$29,500.

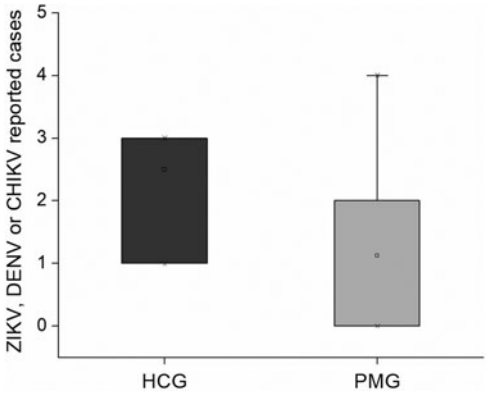

FIG. 2. Boxplot of notified cases within respective areas of the HCG and PMG, obtained during the following seven epidemiological weeks (49 days) after diagnosis of positive mosquitoes and human cases in the city of Foz do Iguaçu, southern Brazil, 2017.

After the vector control, human cases during the following seven epidemiological weeks (49 days) were gathered, and statistical analysis was performed (Table 1). A total of 20 cases were recorded from the HCG and 9 cases for the PMG. When groups were compared, a statistically higher efficacy was observed for the PMG (Mann-Whitney: $z = -2.0185$; $p = 0.043538$) with a 55.0% reduction when compared with the NPDC, the official Brazilian protocol (Fig. 2). Despite relatively high costs of mosquito traps and molecular testing ($\sim$US$35.00 per test) in this study, average costs for dengue patients were even higher, with hospitalization estimated between US$198.00 and US$510.00, and direct medical costs between US$318.00 and US$906.00 (Vieira Machado et al. 2014).

Finally, although results should be carefully interpreted because of the barely significant p-value ($p = 0.043538$), the study herein is the first on-field comparison of vector control effectiveness (and consequently disease spreading) of reporting suspected dengue cases (following official Brazilian protocol) and early molecular diagnosis of trapped mosquitoes. Thus, further studies should be conducted with larger sample sizes, longer time scales, and molecular testing of dead mosquitoes, to fully establish the impact of molecular detection of trapped mosquitoes in the prevention of human arbovirus cases.

Conclusions

The on-field chemical vector control based on molecular arbovirus detection in trapped mosquitoes was shown to be significantly more effective than the traditional reporting of suspected human cases. Such an approach may be routinely applied because the results could be available within 24 h after mosquito trapping, identification, and qPCR testing.

Acknowledgments

The authors thank the employees of the Zoonoses Surveillance Unit, the Itaipu Health Working Group, and the

City of Foz do Iguaçu, Itaipu Binational (Coordination for the Improvement of Higher Education Personnel [CAPES]/Prosup/Unipar).

Author Disclosure Statement

No conflicting financial interests exist.

Funding Information

No funding was received for this article.

References

Blow JA, Mores CN, Dyer J, Dohm DJ. Viral nucleic acid stabilization by RNA extraction reagent. J Virolog Meth 2008; 150:41–44.

Codeço CT, Lima AWS, Araújo SC, Lima JBP, et al. Surveillance of *Aedes aegypti*: Comparison of house index with four alternative traps. PLoS Negl Trop Dis 2015; 9: e0003475.

Donatti JE, Gomes AC. Adultrap: Descrição de armadilha para adulto de *Aedes aegypti* (Diptera, Culicidae). Rev Bras Entomol 2007; 51:255–256.

Fao OIE. WHO, U.N. System Influenza Coordination, UNICEF, The World Bank. Contributing to One World, One Health: A strategic framework for reducing risks of infectious diseases at the animal—human—ecosystems interface. United Nations, 2008:1–68. [updated 2008; cited 2018 Out 12]. Available: http://www.fao.org/3/aj137e/aj137e00.htm

Fauci AS, Morens DM. Zika virus in the Americas: Yet another arbovirus threat. N Engl J Med 2016; 374:601–604.

Glushakova LG, Alto BW, Kim M-S, Hutter D, et al. Multiplexed kit based on Luminex technology and achievements in synthetic biology discriminates Zika, chikungunya, and dengue viruses in mosquitoes. BMC Infect Dis 2019; 19; DOI: 10.1186/s12879-019-3998-z.

Gomes A. de C, Da Silva NN, Bernal RTI, Leandro A. de S, et al. Especificidade da armadilha Adultrap para capturar fêmeas de *Aedes aegypti* (Diptera: Culicidae). Rev Soc Bras Med Trop 2007; 40:216–219.

Gurukumar KR, Priyadarshini D, Patil JA, Bhagat A, et al. Development of real time PCR for detection and quantitation of Dengue Viruses. Virol J 2009; 6:10.

Gutiérrez-Bugallo G, Rodríguez-Roche R, Díaz G, Pérez M, et al. Spatio-temporal distribution of vertically transmitted dengue viruses by *Aedes aegypti* (Diptera: Culicidae) from Arroyo Naranjo, Havana, Cuba. Trop Med Int Health 2018; 23:1342–1349.

Instituto Brasileiro de Geografia e Estatística. Brasil em Síntese: Paraná, Foz do Iguaçu, Panorama [Internet]. IBGE. 2017. Available at: https://cidades.ibge.gov.br/brasil/pr/foz-do-iguacu/panorama

Liotta DJ, Cabanne G, Campos R, Tonon SA. Molecular detection of dengue viruses in field caught *Aedes aegypti* mosquitoes from northeastern Argentina. Rev Latinoam Microbiol 2005; 47:82–87.

Maciel-de-Freitas R, Avendanho FC, Santos R, Sylvestre G, Araújo SC, Lima JBP, et al. Undesirable Consequences of Insecticide Resistance following *Aedes aegypti* Control Activities Due to a Dengue Outbreak. PLoS ONE 2014; 9: e92424.

Mardekian SK, Roberts AL. Diagnostic options and challenges for dengue and chikungunya viruses. BioMed Res Inter 2015.

Medeiros AS, Costa DMP, Branco MSD, Sousa DMC, et al. Dengue virus in *Aedes aegypti* and *Aedes albopictus* in urban areas in the state of Rio Grande do Norte, Brazil: Importance of virological and entomological surveillance. PLoS One 2018; 13:e0194108.

Ministério da Saúde do Brasil. Diretrizes Nacionais para a Prevenção e Controle da Dengue [Internet]. MS. 2009. Available at: www.saude.gov.br/svs

Ministério da Saúde do Brasil. Programa Nacional de Controle da Dengue. Vigilância Epidemiológica. Brasília. 2002, pp. 1–34. Available at: http://bvsms.saude.gov.br/bvs/publicacoes/pncd_2002.pdf

Paramasivan R, Thenmozhi V, Hiriyan J, Dhananjeyan K, et al. Serological and entomological investigations of an outbreak of dengue fever in certain rural areas of Kanyakumari district, Tamil Nadu. Indian J Med Res 2006; 123:697–701.

R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at: https://www.R-project.org/

Samuel PP, Tyagi BK. Diagnostic methods for detection & isolation of dengue viruses from vector mosquitoes. Indian J Med Res 2006; 123 615–628.

Schaffner F, Mathis A. Dengue and dengue vectors in the WHO European region: Past, present, and scenarios for the future. The Lan Inf Dis 2014; 14:1271–1280.

Shukla MK, Singh N, Sharma RK, Barde PV. Utility of dengue NS1 antigen rapid diagnostic test for use in difficult to reach areas and its comparison with dengue NS1 ELISA and qRT-PCR. J Med Virol 2017; 89:1146–1150.

Srisuphanunt M, Sithiprasasna R, Patpoparn S, Attatippaholkun W, et al. ELISA as an alternative tool for epidemiological surveillance for dengue in mosquitoes: a report from Thailand. J Vector Borne Dis 2007; 44:272–276..

Sylvestre G, Gandini M, de Araújo JM, Kubelka CF, et al. Preliminary evaluation on the efficiency of the kit Platelia Dengue NS1 Ag-ELISA to detect dengue virus in dried *Aedes aegypti*: A potential tool to improve dengue surveillance. Parasite Vector 2014; 7:155.

Thenmozhi V, Kabilan L, Philip Samuel P, Dash AP. Short communication: Detection of dengue virus antigens in desiccated mosquitoes: An improved tool for surveillance. Trop Med Int Health 2005; 10:187–189.

Thenmozhi V, Tewari SC, Manavalan R, Balasubramanian R, et al. Natural vertical transmission of dengue viruses in *Aedes aegypt* in southern India. Trans R Soc Trop Med Hyg 2000; 94:507.

ThermoFisher Scientific. MagMAX™-96 Viral RNA Isolation Kit User Guide, Catalog Numbers AM1836, AMB1836-5, 2013. Available from: https://www.thermofisher.com/order/catalog/product/AM1836. Cited 28/04/2018.

Vieira Machado AA, Estevan AO, Sales A, Brabes KCdS, Croda J, Negrão FJ. Direct Costs of Dengue Hospitalization in Brazil: Public and Private Health Care Systems and Use of WHO Guidelines. PLoS Negl Trop Dis 2014; 8:e3104.

Voge NV, Sánchez-Vargas I, Blair CD, Eisen L, Beaty BJ. Detection of dengue virus NS1 antigen in infected *Aedes aegypti* using a commercially available kit. American J Trop Med Hyg 2013; 88:260–266.

Warrilow D, Northill JA, Pyke A, Smith GA. Single rapid TaqMan fluorogenic probe based PCR assay that detects all four dengue serotypes. *J Med Virolog* 2002; 66:524–528.

Weaver SC. Arrival of chikungunya virus in the new world: Prospects for spread and impact on public health. PLoS Negl Trop Dis 2014; 8:e2921.

Woldehanna S, Zimicki S. An expanded one health model: Integrating social science and one health to inform study of the human-animal interface. Soc Sci Med. Pergamon 2015; 129:87–95.

World Health Organization. Arboviruses and human disease. WHO. 1967. Available at: http://apps.who.int/iris/bitstream/10665/40664/1/WHO_TRS_369.pdf

World Health Organization. Arthropod-borne and rodent-borne viral diseases. Report of a WHO Scientific Group. Tech Rep Ser. WHO. 1985; 719:1–116. Available at: www.ncbi.nlm.nih.gov/pubmed/3929480

World Health Organization. Global vector control response 2017–2030. WHO. 2017. Available at: https://www.who.int/vector-control/publications/global-control-response/en/

World Health Organization. Mosquito (vector) control emergency response and preparedness for Zika virus. WHO. 2016. Available at: www.who.int/neglected_diseases/news/mosquito_vector_control_response/en/

Address correspondence to:
André de Souza Leandro
Centro de Controle de Zoonoses da Prefeitura Municipal de Foz do Iguaçu
Avenida Maceió 1511, Jardim Ipê III
Foz do Iguaçu 85869-675
Paraná
Brazil

E-mail: andre.ccz@hotmail.com

Robson Michael Delai
Instituto de Ensino e Pesquisa (IEP) da Fundação de Saúde Itaiguapy
Avenida Araucária, 1734, Vila A
Foz do Iguaçu 85866-010
Paraná
Brazil

E-mail: robson.delai@hmcc.com.br