Project: "Development and Deployment of Low-Cost, Paper-based Zika Diagnostics"

Funded by IDRC - International Development Research Centre

108410-002

Instituto Nacional de Investigación en Salud Pública-INSPI - Ecuador

Research team:

Varsovia Cevallos, Ph.D. – Research Leader
Patricio Ponce, Ph.D. Coordinator/Researcher
Denisse Benitez, M.Sc. Bioinformatics
Victoria Nipaz, Molecular lab technician
Stephany Villota, Ph.D. Collaborating researcher

Final Technical Report

December, 2020

Iquique N° 14-285 y Yaguachi
Teléfono: 022568041 - 022552715 - 022565858
www.investigacionsalud.gob.ec

1. EXECUTIVE SUMMARY

The main aim of the project was to develop a low-cost test for Zika to be deployed first in Ecuador, Brazil, and Colombia. The test was expected to detect the virus in human and mosquito samples and, in turn, to help design effective surveillance programs. In Ecuador, human and mosquito samples were collected in three localities: Borbón, Manta, and Guayaquil. The highest number of cases of Zika infection were registered during 2016 and 2017. By 2018, a rapid reduction of cases of Zika infection (10 cases) caused limitations to obtain infected human and mosquito samples. Nonetheless, our study showed that the implemented low-cost test presented a higher sensitivity to detect the Zika virus in human samples that had been stored for more than two years, compared to the gold-standard technique RT-qPCR. In conclusion, this low-cost diagnostic technology can significantly help improve the diagnostic of the Zika virus and would significantly help to reduce the impact of emerging and re-emerging arboviral diseases in Latin America and other regions of the world.

2. RESEARCH PROBLEM

Zika virus outbreak in the Americas was of global healthcare importance. Ecuador is an endemic area for many mosquito-borne diseases, such as dengue, yellow fever, and lately chikungunya and Zika virus. Ecuador reported a total of 6737 cases from January 2016 to April 2018, when the last case was notified in the country. The co-circulation of three substantially pathogenic arboviruses not only makes differential diagnoses more complicated but also leads to poorly characterized disease manifestations during viral infection. Standard serological approaches, such as antibody detection, are limited in diagnostics due to cross-reactivity in patients that have previously been infected by other flavivirus circulating in the country. As a result, accurate and rapid detection of the Zika virus requires molecular tools, such as PCR. These methods are limited to clinical settings, costly, and not available in remote locations where surveillance and containment are most needed.

Our research aimed to resolve this shortfall. By combining our technology platforms to meet this challenge, our aim was to develop low-cost molecular diagnostics for the virus that can be deployed to the field and operate in remote, low resource environments.

In order to reach the general objective we proposed 3 specific aims:

a) Development of new programmable Zika virus sensors for faster detection, as well as to demonstrate a new class of diagnostic sensor that can distinguish strains of the virus directly. We will also build diagnostics sensors for Dengue and Chikungunya viruses and develop lower cost reagents for molecular reactions (Pardee Lab, lead; Green and Collins Labs).
b) Development of digital microfluidics for sample preparation using digital

Iquique N° 14-285 y Yaguachi
Teléfono: 022568041 - 022552715 - 022565858
www.investigacionsalud.gob.ec

microfluidics. This hardware infrastructure will allow patient/mosquito sample collection, viral lysis, RNA amplification and operation of the Zika virus diagnostics in a single step for users (Wheeler lab).

c) Deployment of the hardware enhanced Zika virus diagnostics to Ecuador, Brazil and Colombia for evaluation of the technology using patient samples and mosquito vectors (Cevallos, Ponce, Castellanos, Pena, Ayres).
    a. Field-Validation Aim I: Patient samples testing.
    b. Field-Validation Aim II: Lab-based device testing
    c. Field-Validation Aim III: Scale-up and field testing.

3. PROGRESS TOWARDS MILLESTONES

Our project had three milestones:

a. To develop and validate a diagnostic tool to detect the Zika virus, which is fast, sensitive, and cheap, which allows differentiation between strains of the Zika virus, and also differentiating it from other viral infections by Dengue and Chikungunya virus.

A technique that presents high sensitivity and specificity to detect the Zika virus was developed as a diagnostic low-cost tool. We used RT-qPCR as the gold-standard to validate our new technology using stored human samples that previously tested positive for the Zika virus. We found that Zika viral charge is lost when human samples are stored. Nonetheless, our diagnostic technique was able to detect a higher number of positive samples compared to RT-qPCR. Furthermore, we used mosquito samples that tested positive for Dengue and Chikungunya virus to determine cross-reaction using Zika sensors. We showed that our diagnostic technique is specific to Zika virus.

b. Develop a diagnostic platform that allows health service providers to collect samples from patients easily and quickly, in order to easily apply the diagnostic test for Zika virus infections in field or intervention sites, without requiring storage or a cold chain distribution system.

Digital microfluidics (DMF) system for sample-to-answer point of care (POC) was developed for the Zika virus. This diagnostic platform was evaluated with the Brazil samples.

c. Implement and evaluate the efficacy, usability, and feasibility of this diagnostic tool in laboratory and field conditions in Ecuador, Brazil, and Colombia.

In Ecuador, the National Institute for Research in Public Health of Ecuador (INSPI) received patient samples from public clinical networks for the diagnostics

Iquique N° 14-285 y Yaguachi
Teléfono: 022568041 - 022552715 - 022565858
www.investigacionsalud.gob.ec

of virus diseases. In Ecuador, the Zika number of cases dropped from approximately 3547 in 2016, to 3183 in 2017, and to 4 cases in 2018 (PAHO_www.paho.org/data/index). Due to the drastic decrease of the Zika virus circulation, validation of the technique under field conditions was not possible. However, under laboratory conditions, this technique presented high sensitivity and specificity.

4. SYNTHESIS OF RESEARCH RESULTS AND DEVELOPMENT OUTCOMES

In Ecuador, our team from the National Institute for Research in Public Health of Ecuador (INSPI) accomplished the following results:

a) Field-Validation Aim I: Patient samples testing.

Human samples that were received as part of the established clinical network were tested for Zika virus using the gold-standard technique RT-qPCR. These tests were run with both positive and negative controls to ensure the correct reaction of the technique. None of the tested samples were positive for the Zika virus.

Additionally, we collected a total of 986 female *Aedes aegypti* mosquitoes from three localities along the coast of Ecuador: Borbón, Manta, and Guayaquil. These samples were grouped in 300 pools which were used to detect three arboviruses (Dengue, Chikungunya, and Zika) by RT-qPCR. Our results showed four mosquito pools positive for the Dengue virus serotype 2 (DENV-2), and two positives for Chikungunya. The percentage of positive pools was 1.33% for DENV-2 and 0.67% for CHIKV. The Minimum Infection Rate (M.I.R.) was calculated as number of positive pools/number of processed individuals x 1,000 (CDC, 2020) and the following values were obtained for DENV-2 M.I.R. 4.06 and CHIKV M.I.R. 2.03. No mosquito samples were positive for the Zika virus.

b) Field-Validation Aim II: Lab-based device testing.

The new molecular tools were deployed in a pilot test at INSPI laboratories in Quito and Guayaquil cities, Ecuador. Sensors for detection of Zika and Chikungunya were developed and available for the essays. Sensors for the Dengue virus were not developed due to technical difficulties.

Zika virus sensors were tested as a pilot study in Guayaquil, Ecuador. Human serum samples, that previously tested positive for the Zika virus, were used to validate the NASBA-cell-free diagnostic technology. Compared to results from gold-standard RT-qPCR, the NASBA-cell-free technique presented higher sensitivity when used to test human serum samples that were stored for two years ($p < 0.01$; Table 1).

Iquique N° 14-285 y Yaguachi
Teléfono: 022568041 - 022552715 - 022565858
www.investigacionsalud.gob.ec

Table 1. Human samples testing positive for the Zika virus. Human serum and culture supernatant samples were analyzed separately. Two techniques were used (RT-qPCR and NASBA-cell-free reactions) to detect Zika virus.

Sample	Number of samples	qRT-PCR Positive (%)	NASBA Positive (%)	*P* value
Serum	92	11 (12.0)	50 (54.3)	< 0.01
Supernatant	4	2 (50.0)	1 (25.0)	0.64
TOTAL	**96**	**13 (13.5)**	**51(53.1)**	**< 0.01**

Our results showed a clear reduction in the viral load of the stored samples, as the Zika virus was undetected in near 50% of them. However, the results from the NASBA-cell-free technique clearly showed a higher sensitivity of our diagnostic technique compared to the gold-standard RT-qPCR.

Furthermore, we used the six mosquito pools that tested positive for Dengue and Chikungunya, and 21 pools that tested negative for Zika, Dengue or Chikungunya to determine the specificity of the NASBA-cell-free technique using Zika sensors. Our results showed that none of the tested samples were positive for Zika virus (Figure 1). These results show high specificity of our technique as there was no cross-reaction with the other two arboviruses.

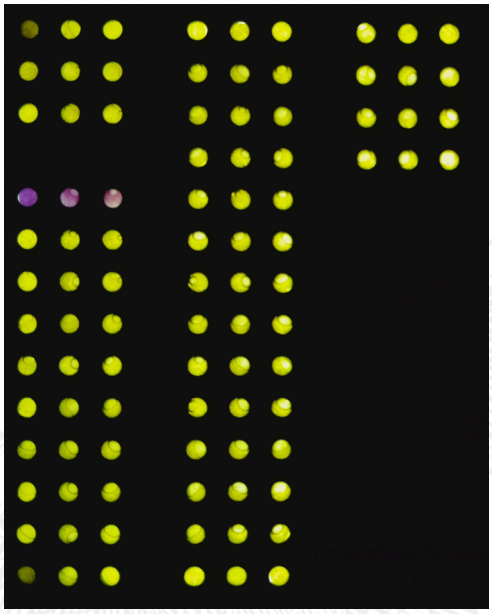

Figure 1. Results from the detection of the Zika virus using the NASBA-cell-free technique in mosquito samples. Positive control changed color (purple) to confirm the correct reaction of the technique. Samples run in triplicate, all samples negative to Zika virus (yellow)

Iquique N° 14-285 y Yaguachi
Teléfono: 022568041 - 022552715 - 022565858
www.investigacionsalud.gob.ec

Taken together, our results from human and mosquito samples demonstrate that the NASBA-cell-free technique using Zika sensors presents high sensitivity and specificity.

c) Field-Validation Aim III: Scale-up and field testing.

During the final phase of the project, optimized molecular tools and hardware manufactured at the scale necessary to support extensive testing in Latin America. However due to the drastic decrease of the Zika virus circulation in Ecuador, validation of the technique under field conditions was not possible.

5. METHODOLOGY

Field-validation of this low-cost diagnostic technology was initially planned to be tested in blood samples from infected patients that sought medical attention. Nonetheless, due to the unforeseen reduction of Zika cases registered since 2018, it was necessary to use stored human samples that tested positive for the Zika virus when first processed back in 2017. A total of 96 human Zika positive samples that were stored at INSPI in Quito and Guayaquil were tested.

Detection of the Zika virus was done using the gold-standard technique RT-qPCR with primers and probes reported by Lanciotti and collaborators in 2008 (Zik1086, Zik1162c, probe Zik1107FAM). These results were compared to the ones obtained with our diagnostic technique: a combination of nucleic-acid-sequence-based amplification (NASBA) and cell-free reactions (pCOLADuet-Switch3B-LacZ and pET15-Trigger3). Blinded samples were used to test for true negative/positive samples as well as false negative/positive samples, results were compared to those generated using qPCR.

Mosquito detection essays:

Adult mosquitoes were collected with a Prokopack aspirator from three localities along the coast of Ecuador: Borbón, Manta, and Guayaquil. The female *Aedes aegypti* mosquitoes were collected and stored in RNAlater for molecular detection of Zika virus using the previously described techniques. Mosquitoes were processed individually or in pools depending if they were fed or unfed. The criteria for choosing the mosquito samples to be processed depended on reports of possible arboviral infections in the proximity of the sampling sites. The samples were grouped in 300 pools which were used to detect three arboviruses (Dengue, Chikungunya, and Zika) by RT-qPCR.

Iquique N° 14-285 y Yaguachi
Teléfono: 022568041 - 022552715 - 022565858
www.investigacionsalud.gob.ec

6. PROJECT OUTPUTS

Our results showed a clear reduction in the viral load of the stored human samples, as the Zika virus was undetected in near 50% of them. However, the results from the NASBA-cell-free technique using Zika sensors clearly showed a higher sensitivity of our diagnostic technique compared to the gold-standard RT-qPCR. This outcome is reassuring as our main objective was to develop a faster sensor for the Zika virus as a low-cost test.

We tested mosquito samples that were positive for other arboviruses (Dengue and Chikungunya), as well as samples negative for arboviruses by our NASBA-cell-free technique using Zika sensors. None of the tested samples were positive for the Zika virus, and there was no cross-reaction with the other two arboviruses. Hence, our new technique presents a high specificity towards the detection of the Zika virus.

Furthermore, this project contributed to knowledge sharing with society through training experiences and conference presentations in the First International Symposium on Low Cost, Paper-based Zika Diagnostics on 23rd March 2018 (Appendix 1, 2). Two undergraduate students from "Universidad Central del Ecuador" were trained in synthetic biology techniques and interpretation of results. During the "First Genetic and Genomics Ecuadorian Symposium" held at "Universidad San Francisco de Quito" in April 2019, Denisse Benítez shared a project update with the presentation entitled "Development and deployment of a new low-cost technique for the diagnosis and detection of the Zika virus in Ecuador". The presentation focused on the development of the diagnostic tool performed by the Toronto team, the work methodology, and the contribution of INSPI to the project. This event counted with more than twenty speakers from the Genetics and Genomics Ecuadorian Network (ReGG) and gathered more than 150 participants. Additionally, during the "Annual Cycle of Scientific Conferences" held at INSPI – Quito in September 2019, Denisse Benítez and Victoria Nipaz presented a summary of the advances of the project. Finally, the results from the project were presented by Stephany Villota in a virtual conference entitled "Development and implementation of a new low-cost technique for the diagnosis and detection of the Zika virus in Ecuador in the period 2017-2020" in December 2020. The conference focused on the final outcomes of the project and a detailed explanation on how this new technique works by the implemented synthetic biology techniques.

Sensors have been designed for the detection of the Chikungunya virus by the Canada team. The detection of Chikungunya virus in human and mosquito samples collected in Ecuador has yet to be done. Human serum samples from patients that tested positive for Chikungunya were obtained from INSPI Guayaquil. These samples have been

Iquique N° 14-285 y Yaguachi
Teléfono: 022568041 - 022552715 - 022565858
www.investigacionsalud.gob.ec

stored for almost two years. Our lab team will use these samples to validate our NASBA-cell-free technique using Chikungunya sensors compared to the gold-standard RT-qPCR. Results are expected to be obtained by the end of March 2021. These results will be of highly importance as there have been two confirmed Chikungunya cases in Ecuador during 2019. In the unfortunate event of a Chikungunya outbreak, our technique could be implemented to facilitate a quick and sensitive diagnosis.

A multi-country publication is being prepared and will be submitted by the end of March 2021: *"Adaptive, Low-cost Gene Circuit-based Sensors and Hardware: A Patient-trial for Cell-free, Paper-based Zika and Chikungunya Virus Diagnostics"*

7. PROBLEMS AND CHALLENGES

Our major challenge was the unexpected decrease in the Zika virus circulation. This project was planned for a three-year period ending by 2020. In Ecuador, the peak of the Zika virus circulation was between 2016 and 2017, and a sudden decrease in reported cases was seen by 2018. It was after this time period that our fieldwork was planned to start. Due to this situation, we were forced to use stored human samples to cover most of the aims of the project. Regardless of our efforts, we were not able to fulfill the testing of the equipment under field conditions.

8. ADMINISTRATIVE REFLECTIONS AND RECOMMENDATIONS

We thank IDRC for all the help to get funds available in Ecuador. In general, funds availability was appropriate and allowed us to fulfill the planned activities in the project.

We appreciate the help from the financial and technical assistants with all the paperwork and follow up throughout the project.

Iquique N° 14-285 y Yaguachi
Teléfono: 022568041 - 022552715 - 022565858
www.investigacionsalud.gob.ec

Appendix 1

Appendix 2

Iquique N° 14-285 y Yaguachi
Teléfono: 022568041 - 022552715 - 022565858
www.investigacionsalud.gob.ec