Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz
Instituto Aggeu Magalhães

Final Technical Report
(Extension from April 2020 to November 2020)

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

Grant. No. 108410-001

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1. Synthesis

We proposed to create a deployable, low cost platform for the diagnosis and surveillance of the Zika virus using cell-free synthetic gene networks. We have developed two disruptive technologies that promise to dramatically lower the cost and technical barriers to the development of diagnostics. Specifically, this includes a novel, cell-free method to deploy poised synthetic gene networks to the field and new programmable RNA sensors called toehold switches. The Canadian team has recently used these technologies to create programmable Zika virus diagnostics that can be developed rapidly, inexpensively and have a low manufacturing cost per sensor. Here we extend our proof-of-concept work to create field-ready molecular tools for the Zika virus and also chikungunya virus. Due to the COVID-19 pandemic, we have requested an extension of the project until November 2020, to successfully complete the planned activities. During this period, we finished carried out the final experiments aimed at validation and proof of concept of the molecular sensors for the diagnosis of Zika and chikungunya viruses in patient samples using a total of 268 samples for Zika and 65 patient samples for chikungunya virus. The diagnostic accuracy was 98.5% and 98.46% for Zika and chikungunya, respectively. This project paved the way for approval an additional grant (IDRC PROJECT ID 109434) that will aims to use the same technology against SARS-CoV-2, the coronavirus underlying COVIV-19. The availability of these portable diagnostic tools has great potential help to prevent the spread of the disease within populations and improve patient outcomes both in Latin America and globally as the outbreak spreads.

Objectives

1) Diagnostic: Development of faster sensors for the Zika virus (12 sensors), a new unpublished class of diagnostic sensor that can detect strain-specific, single nucleotide polymorphism (SNPs) for the direct discrimination of Zika viral strains (24 sensors), and Chikungunya (24 sensors) viruses to provide more comprehensive diagnostic tools for health care providers. We will also

development low cost alternatives to the commercial biochemical products that run the molecular reactions, with the aim of ultimately reducing the cost even further.

2) Technical: Development of digital microfluidic system for sample-to-answer point of care (POC) assay delivery. Team expertise in this area will embed our molecular tools within a hardware infrastructure that will allow patient sample collection, viral lysis, RNA amplification and operation of the Zika virus diagnostics in a single step for users.

3) Field validation: Deployment of our hardware-enhanced Zika virus diagnostics to team members in Ecuador, Brazil and Colombia for evaluation of the technology using patient samples and mosquitos from surveillance programs under both laboratory and field conditions.

Specifically, the Brazilian research team led by Dr. Lindomar Pena was responsible for the objective 3- Field validation-using both clinical and mosquito samples.

2. The research problem

As part of national reference laboratories or other well-established clinical networks, team members tested incoming samples using conventional qPCR to determine the presence or absence of the Zika virus. These tests are run with both positive and negative controls. In a parallel and blinded process, the same samples were tested using the existing toehold switch-based diagnostics by the Canadian team. With the guidance of team members who developed the system, this work will be used to test and optimize the molecular conditions for diagnosing patient samples on-chip in the subsequent months. They have successfully tested our system on blood plasma from an infected macaque and expect human samples to similarly work well. All sample collection and testing have be done with patient consent and in accordance with national and institution ethical policies and regulatory approvals have been granted. Thus, team members from Brazil have collected and tested humans and mosquito samples using RT-PCR as a gold standard and use these results to latter validate the new technology developed by the Canadian team.

3. Research findings

Human resources training

During the extension, we continued the training of one PhD student- Mr.Severino Jefferson Ribeiro da Silva.

Severino Jefferson Ribeiro da Silva finished his Masters at the Fiocruz´s Biosciences and Biotechnology Applied to Health Graduate Program on February 27th, 2018 and begun his PhD in the same program in April 2019. The title of his PhD dissertation project is "*Validation of "Point-of-Care" Diagnostic Platforms for Molecular Diagnosis of the Zika Virus*". His PhD work is directed by me and his as the co-advisor is Dr. Keith Pardee, PI from this project. He was approved by the PhD Program in November 2018 and begun his studies in April 2019, working on this project.

During the extension, we finished validating the clinical samples and also spent time written and submitting research articles since Brazil had several quarantine and lock downs in the period.

Below is a list of the 10 papers we have written and published last year. The first 4 papers are in collaboration with the Canadian team. The extension of the grant was very helpful to support us to do this work.

1. DA SILVA, SEVERINO JEFFERSON RIBEIRO ; PARDEE, KEITH ; BALASURIYA, UDENI B. R. ; Pena, Lindomar . Development and validation of a one-step reverse transcription loop-mediated isothermal amplification (RT-LAMP) for rapid detection of ZIKV in patient samples from Brazil. Scientific Reports, v. 11, p. XXX, 2021.
2. MATTHEWS, QUINN ; DA SILVA, SEVERINO JEFFERSON RIBEIRO ; NOROUZI, MASOUD ; PENA, LINDOMAR JOSÉ ; PARDEE, KEITH . Adaptive, diverse and de-centralized diagnostics are key to the future of outbreak response. BMC BIOLOGY, v. 18, p. xxx, 2020.
3. SILVA, SEVERINO JEFFERSON RIBEIRO DA ; PARDEE, KEITH ; Pena, Lindomar . Loop-Mediated Isothermal Amplification (LAMP) for the Diagnosis of Zika Virus: A Review. Viruses-Basel, v. 12, p. 19, 2020.

4. SILVA, SEVERINO JEFFERSON RIBEIRO DA ; SILVA, CAROLINE ; GUARINES, KLARISSA ; MENDES, RENATA ; PARDEE, KEITH M. ; KOHL, ALAIN ; Pena, Lindomar . Clinical and Laboratory Diagnosis of SARS-CoV-2, the Virus Causing COVID-19. ACS Infectious Diseases, v. xx, p. xx-xx, 2020.

5. SILVA, SEVERINO JEFFERSON RIBEIRO DA ; MAGALHÃES, JURANDY JÚNIOR FERRAZ DE ; Pena, Lindomar . Simultaneous Circulation of DENV, CHIKV, ZIKV and SARS-CoV-2 in Brazil: an Inconvenient Truth. One Health, v. 12, p. 100205, 2021.
6. DA SILVA, SEVERINO JEFFERSON RIBEIRO ; DE MAGALHÃES, JURANDY JÚNIOR FERRAZ ; MENDES, RENATA PESSÔA GERMANO ; PENA, LINDOMAR JOSÉ . Has Zika Virus Established a Sylvatic Cycle in South America?. ACTA TROPICA, v. xx, p. 105525, 2020.
7. DA SILVA, SEVERINO JEFFERSON RIBEIRO ; DA SILVA, CAROLINE TARGINO ALVES ; MENDES, RENATA PESSÔA GERMANO ; Pena, Lindomar . Role of Nonstructural Proteins in the Pathogenesis of SARS-CoV-2. JOURNAL OF MEDICAL VIROLOGY, v. xx, p. xx, 2020.
8. SILVA, SEVERINO JEFFERSON RIBEIRO DA ; GERMANO MENDES, RENATA PESSÔA ; ALVES DA SILVA, CAROLINE TARGINO ; LORUSSO, ALESSIO ; KOHL, ALAIN ; Pena, Lindomar . Insights into SARS-CoV-2, the Coronavirus Underlying COVID-19: Recent Genomic Data and the Development of Reverse Genetics Systems. JOURNAL OF GENERAL VIROLOGY, v. xx, p. xxx, 2020.
9. MAGALHÃES, JURANDY JÚNIOR FERRAZ DE ; MENDES, RENATA PESSOA GERMANO ; SILVA, CAROLINE TARGINO ALVES DA ; SILVA, SEVERINO JEFFERSON RIBEIRO DA ; GUARINES, KLARISSA MIRANDA ; Pena, Lindomar . Epidemiological and clinical characteristics of the first 557 successive patients with COVID-19 in Pernambuco state, Northeast Brazil. Travel Medicine and Infectious Disease, v. 38, p. 101884, 2020.
10. SILVA, SEVERINO JEFFERSON RIBEIRO DA ; PENA, LINDOMAR J. . A word of caution in interpreting COVID-19 diagnostics tests. JOURNAL OF MEDICAL VIROLOGY, v. xx, p. xxx-xxx, 2020.

Research activities

ZIKV DIAGNOSTIC PATIENT TRIAL

Following molecular and hardware training on-site in Latin America, we evaluated the performance of the ZIKVdiagnostic platform using cultured viruses (analytical specificity and sensitivity) and patient samples (diagnostic performance). A standardized diagnostic workflow was used in all experiments where, following RNA extraction, samples were tested using ZIKV virus-specific NASBA and paper-based cell-free reactions (Fig. 1).

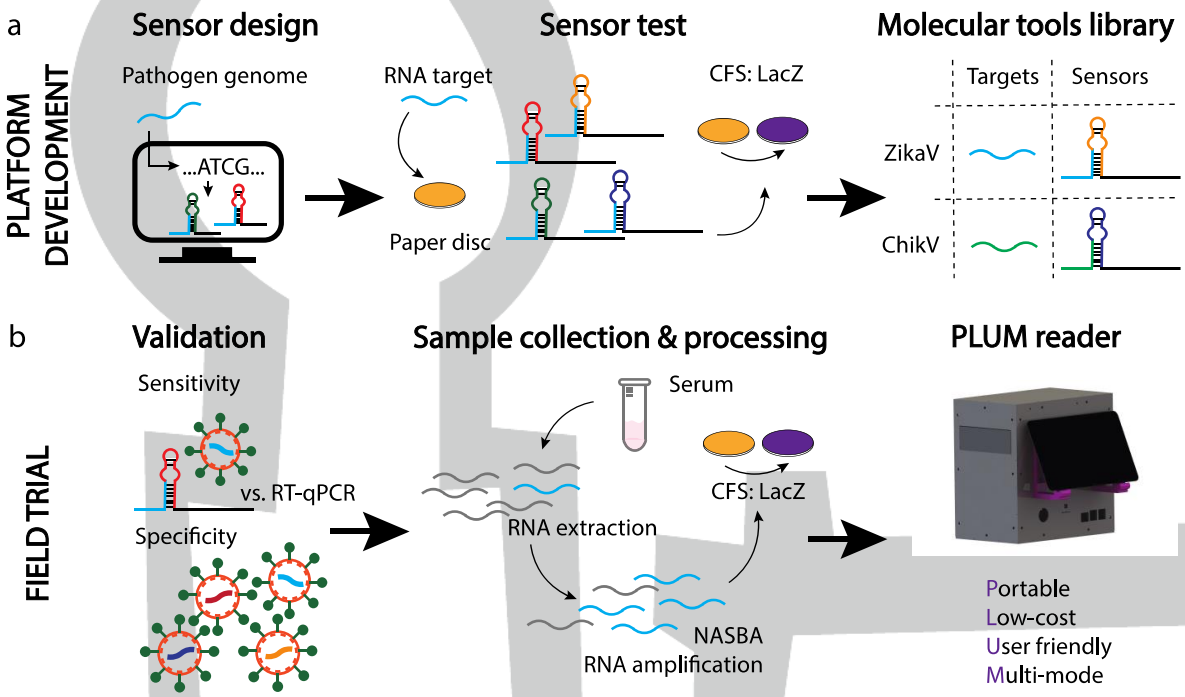


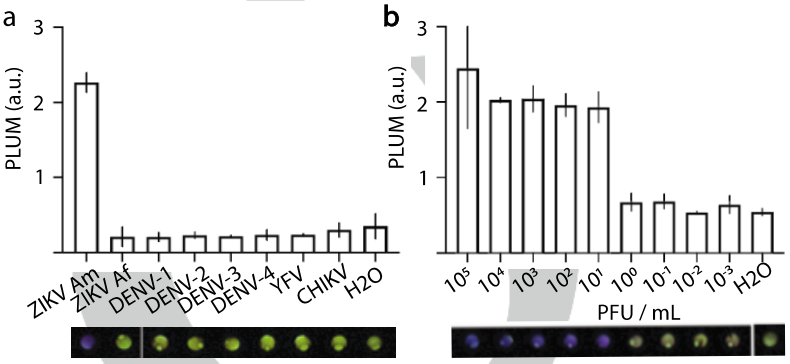
Fig. 1. Schematic of the paper-based diagnostic platform.

The PLUM reader was used exclusively here and provided early and unbiased quantification of the colorimetric responses in real time. In parallel, as a gold-standard comparison, all samples were tested the same day using the RT-qPCR protocol for the ZIKV developed at the US Centers for Disease Control (CDC). Patient samples were assayed and analyzed considering the Cq value of ≤ 38.0 for positivity to ZIKV. Samples with Cq value of > 38 were determined as negative.

We began the characterization of the diagnostic platform by testing the analytical specificity for the American strain of the ZIKV against a panel of seven endemic arboviruses in Brazil that could, in practice, be found in patients with similar symptoms to those associated with the ZIKV. This included the chikungunya (CHIKV), yellow fever (YFV), along with the four serotypes of the dengue virus (DENV 1-4). We also evaluted strain specificity with addition of the African strain of ZIKV (ZIKV Af). As can be seen by eye, a positive signal (purple color) was only detected in the presence of the American strain of the ZIKV(Fig. 2a bottom). The colorimetric response of reactions were quantified using the PLUM reader, with these results directly matching those from the parallel RT-qPCR assay (Fig. 2a graph).

We next evaluated the analytical sensitivity of the paper-based ZIKV test. Here, we performed serial dilutions of the ZIKV followed by RNA extraction. The results demonstrate that the sensitivity of the paper-based diagnostic platform is equivalent to the gold standard RT-qPCR assay, with detection of the virus down to 10^1 PFU/mL (Fig. 2b).

The resulting PLUM-based analysis allowed for the differentiation of positive from negative samples as early as 75 minutes with an accuracy of 98.51 % (± 2.29 %). A total of 268 patient samples were analyzed using our diagnostic platform in parallel with RT-qPCR (Fig. 2c). In comparison to the RT-qPCR assay[24], we detected four false negative and zero false positive samples, which translates to a calculated sensitivity, specificity and diagnostic accuracy of 94.52%, 100% and 98.51%, respectively.



c

Zika analysis	RT-qPCR +	RT-qPCR -
PLUM +	69	0
PLUM -	4	195
Diagnostic Sensitivity	94.52 %	
Diagnostic Specificity	100 %	
Diagnostic Accuracy	98.51 %	

Fig. 2. Performance of the diagnostic platform with Zika virus in Latin America.

DIAGNOSTIC MODULARITY

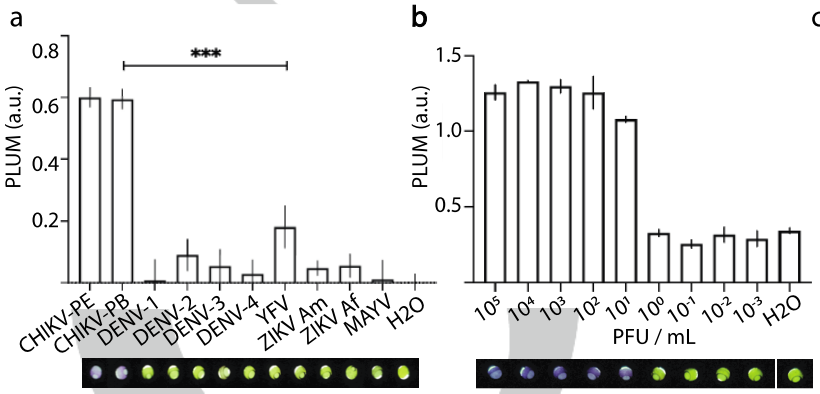
Given the success of the ZIKV patient trial on-site in Latin America, we sought to demonstrate the versatility of the PLUM reader with programmable gene circuit-based diagnostics. For the next diagnostic demonstration, we chose the mosquito-borne CHIKV, which has symptoms overlapping with the ZIKV. Like the ZIKV, chikungunya originated in Africa and has recently arrived in Latin America; with a sustained and ongoing spread worldwide. Symptoms

include fever and soreness, and, while most patients recover within weeks, severe joint pain that can last for months.

As with the ZIKV diagnostic, we began with the computational design of 48 toehold switches targeting various regions of the CHIKV RNA genome. Each toehold switch candidate was linked to the LacZ reporter and tested for detection of the corresponding synthetic target RNA sequence. The top performing sensor was then optimized for analytical sensitivity using several combinations of NASBA primers specific to the region of the targeted chikungunya sequence. The combined NASBA and toehold switch-based test was able to detect the target synthetic RNA down to the clinically relevant range of 10^3 molecules/μL.

With the molecular components validated, they were distributed to the field site in Recife, Brazil. Here the paper-based test was evaluated for diagnostic capacity using cultured CHIKV strains (Paraiba, PB and Pernambuco, PE states) and, tested for analytical specificity against a panel of eight off-target (including the related *Alphavirus* Mayaro virus - MAYV) endemic arboviruses. As before, the CHIKV diagnostic test provided 100% analytical specificity for target strains (CHIKV PE, PB; Fig. 3a). Similarly, when evaluated for analytical sensitivity using titrated CHIKV (strain PE2016-480), the paper-based assay provided detection down to 10 PFU/mL (Fig. 3b). While the RT-qPCR assay was more sensitive than the paper-based test, this did not seem to limit performance with patient samples. Based on the analytical sensitivity data of PLUM, a CHIKV-specific threshold value (0.08 a.u. above background) was established at 75 min. Diagnostic accuracy of the CHIKV test was calculated to

be of 98.46% in a double-blinded, 65-patient study with only one false negative and had the diagnostic sensitivity and specificity of 92.31% and 100% respectively (Fig. 3c).



ChikV analysis	RT-qPCR +	RT-qPCR -
PLUM +	12	0
PLUM -	1	52
Diagnostic Sensitivity	92.31 %	
Diagnostic Specificity	100 %	
Diagnostic Accuracy	98.46 %	

Fig. 3. Performance of the diagnostic platform with Chikungunya virus in Latin America.

The manuscript reporting these results has been positively reviewed and may be accepted for publication very soon.

4. Project implementation and management

The extension of the project allowed us to successfully complete the project goals and contributed a lot for implementation of technologies never used before in Brazil. The financial support provided by the project was very important for this work, especially that Brazilian science has been severely impacted by the economic crisis and now the pandemics.

The Brazilian team has also partnered with the Pernambuco State Diagnostic Lab (LACEN), which is the Lab that receives and test samples from

patient seen at the public health units and hospitals in Brazil (Fiocruz is a reference lab and does confirmatory tests and more sophisticated assays such as PRNT).

This project paved the way for approval an additional grant (IDRC PROJECT ID 109434) that will aims to use the same technology against SARS-CoV-2, the coronavirus underlying COVIV-19. The collaboration with the Canadian team has been very successful and will certainly expand it to new areas.

5. Project outputs and dissemination

As deliverables, the Brazilian team has achieved:

1) Validate a low cost and portable approach for diagnosing Zika virus in patient and mosquito samples;
2) Field-based testing of Zika virus diagnostics in Brazil;
3) Evaluate the capability of the assay for chikungunya detection in portable format;
4) Train human resource on molecular biology and diagnostics techniques.
5) Publish high impact papers.
6) Consolidate the collaboration with the Canadian and other team members.

6. Impact

The project has contributed to bring a novel technology to Brazil: the use of synthetic biology for diagnostics. All the team members have been positively impacted by the project and certainly will be better prepared to use this technology for other pathogens, such as SARS-CoV-2, the causative agent of COVID-19.

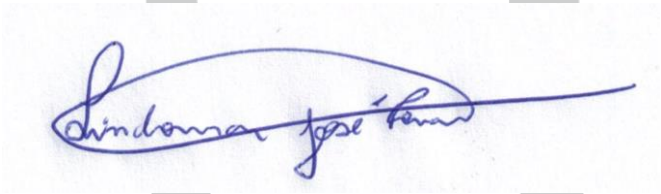
The triple epidemics caused by dengue virus (DENV), chikungunya virus (CHIKV) and Zika virus (ZIKV) represent a serious threat to health systems in several parts of the world, especially in tropical countries where mosquitoes from the genus Aedes are widespread. Similar to other developing countries, Brazil has been facing several challenges for Covid-19 diagnosis, especially with regarding the lack of supplies and equipments required for SARS-CoV-2 detection. Moreover, many laboratories redirected all their efforts to diagnose SARS-CoV-2,

including the reference arbovirus laboratories, which had a direct impact on arbovirus detection. In this context, the Brazilian government has increased the number of testing for Covid-19 as the pandemic advance, but the rate of testing is still much lower compared to other countries.

This public health crisis highlighted the need for rapid and low-cost testing that can be deployed beyond the reach of centralized clinical diagnostic labs. Such centralized labs use RT-qPCR for the detection of pathogens, which, while tremendously sensitive and specific, requires specialized laboratory equipment and expertise to perform. The result is a network of diagnostic hubs that can be difficult to scale during an outbreak, often leading to bottlenecks in testing. This was the case in the hardest hit country, Brazil, where RT-qPCR based diagnostics for the ZIKV was provided by five centralized national reference laboratories, which led to limited access and delays in results. The circumstance was worsened by overlapping clinical symptoms of the ZIKV with other endemic arboviruses, cross-reactivity in antibody tests and a lack of portable antigen tests. This shortfall in diagnostic capacity led to calls for molecular diagnostics that can be used at the point-of-care (POC) and motivated the development of several new ZIKV diagnostic technologies, many of which came from the field of synthetic biology. We see emerging diagnostics, like the paper-based test developed here, as having tremendous near-term potential to augment existing RT-qPCR capacity, improve equity in the access to health care and to aid the response to public health crises.

7. Recommendations:

We would like to thank the IDRC for financial support and for providing all the assistance needed to successfully complete this grant.



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