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Development of Loop-Mediated Isothermal Amplification Assay for Rapid Detection of Dengue Virus in *Aedes aegypti* (Diptera: Culicidae) Larvae from Cuba

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ABSTRACT

Dengue fever is an arthropod-borne disease of great importance worldwide due to the number of cases, epidemics and geographical extent of its vectors. *Aedes aegypti* (Linnaeus) mosquito is its main vector and its chemical control is the only viable alternative to stop dengue outbreaks since there is not an effective dengue vaccine. Even though surveillance systems allow a rapid detection of dengue cases; sometimes it is not enough to prevent outbreaks due to asymptomatic cases that cannot be detected. Nine pools of field-caught *Ae. aegypti* larvae were collected in Havana, Cuba. They were analyzed by reverse transcription–polymerase chain reaction (RT-PCR) and loop-mediated isothermal amplification (RT-LAMP) assays to detect dengue virus. From them4 out of nine were positive by RT-PCR and 9 were positive by RT-LAMP with and without RNA extraction step, respectively. RT-LAMP assay, with and without RNA extraction step, was able to detect more positive samples than RT-PCR standing out as a powerful tool to dengue surveillance in mosquito populations for early prediction of outbreaks.

INTRODUCTION

According to the clinical Guide of Dengue, dengue with or without warning signs (D) and Severe Dengue (SD) are caused by the four antigenically related Dengue Virus (DENV) serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) from *Flaviviridae* family, genus *Flavivirus* [1]. It is one of the most important challenge for public health worldwide, which has been increased in magnitude due to the extended geographical distribution of its main vector *Aedes aegypti* (Linnaeus), demographic variations including rising and unplanned urbanization and unsuccessful mosquito control in most of dengue-endemic areas in the world [2,3]. Annually, dengue fever produces more than 100 million cases of classic dengue fever and around 450,000 cases of hemorrhagic fever [2,3]. In Cuba, most of cases caused by DENV-2 were severe and clinically classified as Dengue Hemorrhagic Fever/Dengue Shock Syndrome (DHF/DSS), and 158 people died [4]. The low infestation indexes of *Ae. aegypti* and

the information obtainable of passive surveillance system showed no dengue transmission between 1981 and the end of 1996, in the country. In 1997, several kinds of factors in the municipality of Santiago de Cuba favored re-infestation and the increase of vector that spread throughout the country producing annual dengue outbreaks up to now [5]. Recently, vertical transmission of DENV was showed in *Ae. aegypti* larvae caught in a municipality of Havana, Cuba [6].

Detection of DENV in human and mosquito samples during low and high transmission periods is essential for clinical and epidemiological research, as well as an earlier warning system to prevent dengue outbreaks. Development of molecular biology provides new and powerful tools to prevent or control transmission, since there is not an effective dengue vaccine. Some molecular techniques used to detect genomic sequence of viral RNA by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) [2,7] and real-time quantitative RT-PCR (qRT-PCR) [8,9] are highly useful to detect DENV in human sera and mosquito samples. However, important obstacles such as the requirement of nucleic acid extraction and costly instruments, time-consuming and high level of skill have restricted their application especially in resource-limited laboratories and rural health care settings.

Loop-Mediated Isothermal Amplification (LAMP) assay was developed to diagnose infectious diseases, essentially, in developing countries [10] due to simplicity, ruggedness, low cost, high specificity and sensitivity and DNA or RNA isolation step is not required. It has been described for several pathogens such as Dengue, in clinical samples [11-13], but so far it has not been used in *Ae. aegypti* larvae. In this study, we reported the attainment of detection of dengue viruses in field-caught of *Ae. aegypti* larvae through the use of the LAMP assay in Cuba. Results from LAMP were compared using conventional RT-PCR as a gold standard.

MATERIALS AND METHODS

Two hundred seventy *Ae. aegypti* larvae were collected in three municipalities from January to March 2018, in Havana, Cuba. Samples were morphologically identified and sorted in the nine pools of thirty larvae in Vector Control Department at Institute of Tropical Medicine Pedro Kouri. They were preserved at - 80°C until being tested. Larvae were homogenized using a TissueLyser Homogenizer (QIAGEN, Germany) at a frequency of 30 cycles/s during 1 minute, subsequently were centrifuged at 4°C to 20,000 x g for 10 minutes and 140µl out of 250µl of supernatant was taken to RNA isolation using the QIAamp Viral RNA Mini kit (QIAGEN, Germany) according to the manufacturer's protocol. The rest of crude mosquito lysate and RNA eluted were stored at -80°C until to be used. Detection of DENV serotypes was performed using a conventional RT-PCR [7].

Table 1: RT-LAMP primers used for the rapid detection of DENV [12].

Primer[ab]	Sequence (5'→3')
F3/134	CAAACCGTGCTGCCTGT
F3/2	TGAGTAAACTATGCAGCCTGT
B3/123	ACCTGTTGATTCAACAGCACC
B3/4	ACCTGTTGGATCAACAACACC
FIP/123	AGGGGTCTCCTCTAACCRCTAGTCTTTCAAACCRTGGAAGC TGTACGC
FIP/4	AGGGGTCTCCTCTAACCRCTAGTCTTTTTTGCCACGGAAGCT GTACGC
BIP/123	ACAGCATATTGACGCTGGGARAGACGTTCTGTGCCTGGAAT GATGCTG
BIP/4	ACAGCATATTGACGCTGGGARAGACGCTCTGTGCCTGGATT GATGTTG
BLP/123 4	CAGAGATCCTGCTGTCTC

[a]F3 forward outer primer, B3 backward outer primer, FIP forward inner primer, BIP backward inner primer, BLP backward loops primer.
[b]Number (1-4) after the slash (/) represents DENV serotype (e.g. F3/134, forward outer primer for DENV-1, DENV-3 and DENV−4).

RT-LAMP assay used to DENVs diagnosis in *Ae. aegypti* larvae was performed with a total of 25µL final reaction containing 5 pmol/L each of innerprimers (FIP/123, FIP/4, BIP/123, and BIP/4), 25 pmol/L each of outer primers (F3/134, F3/2, B3/123, and B3/4) and 25 pmol/L of loop primer (BLP/1234) described below (Table 1), 1X Buffer Reaction Mix (0.1% Tween 20, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 10mM KCl and 20mM Tris-HCl, pH 8.8), 1.4mM deoxynucleotide triphosphates, 0.8M betaine, 6mM $MgSO_4$, 16U of *Bst* DNA polymerase (GENEON, Germany), 0.125U of avian myeloblastosis virus reverse transcriptase(Promega, USA), 3µL of RNA template and a final reaction volume of nuclease free water adjusted to 25µL.

The reaction was incubated to 63°C for 80 min and subsequently, inactivated at 80°C per 5 min. Finally, it added 1 µL of SYBR Green I dye (Sigma-Aldrich, USA) to each tube where it observed, through naked-eye, with a fluorescent emission in positive samples.

The Minimum Infection Rate (MIR) was calculated as: MIR = (number of positive pools ÷ total number of larvae tested)x 1000.

RESULT AND DISCUSSION

Figure 1: RT-PCR assay of the isolation of viral RNA in pools of *Ae. aegypti* larvae. Amplification with specific primers for TS1, TS2, TS3 and TS4 type in positive control with four DENV serotypes (PC lane) and RNA isolation of larvae pools (lanes 2, 3, 4 and 5): 119 bp (DENV-2), 482 pb (DENV-1), 290 bp (DENV-3) and 392 bp (DENV-4); RNA of larvae pools (lanes 1, 6, 7, 8 and 9), clinical sample positive to Zika virus (Tube ZIKV) and non-template control (Tube NTC) was negative to DENV; lane M, stair marker of 100 bp. DNA sizes are given in base pairs.

This work has demonstrated the application of RT-LAMP assay to detect DENV in mosquitoes from nine pools analyzed, four were positive for DENV by RT-PCR and all of them were positive by RT-LAMP, with and without RNA extraction step, respectively. Those results revealed how RT-LAMP (with or without RNA extraction step) was able to detect more positive samples than RT-PCR being consistent with other articles where RT-LAMP sensitivity was 10-100 folds higher than conventional RT-PCR to DENV and 10000 fold higher than qRT-PCR to ZIKV [11,14]. Both RT-LAMP assays (with and without RNA extraction step) had got the same sensitivity. However, RT-LAMP assay without RNA extraction step presented a lower intensity of fluorescence in some samples, probably due to low concentration of RNA in crude mosquito lysates (Figure 2). MIRs gotten by RT-PCR (14.81) and RT-LAMP (33.33) assays in these samples were consistent with those reported in Singapore and Cuba, by RT-PCR assay [6, 15]. Two clinical samples (one positive to ZIKV and another to HCV by qRT-PCR) amplified neither RT-PCR nor RT-LAMP assays for dengue what proved the specificity of both techniques. These results are consistent with previous studies about the specificity of the LAMP assay for DENV using other flavivirus as negative control [11,12,16,17]. In this study was also confirmed natural DENV vertical transmission of four serotypes in Havana city (Figure 1) being consistent with previous reports where *Ae. aegypti* larvae from Cuba were positive to DENV-3 [6]. Although the diagnosis of some pathogens such as ZIKV has previously been described in mosquitoes [18], at present, this is the first report of DENV detection in wild *Ae. aegypti* larvae by RT-LAMP assay, with purified RNA of the samples (Figure 2) and in crude mosquito lysates (Figure 3).

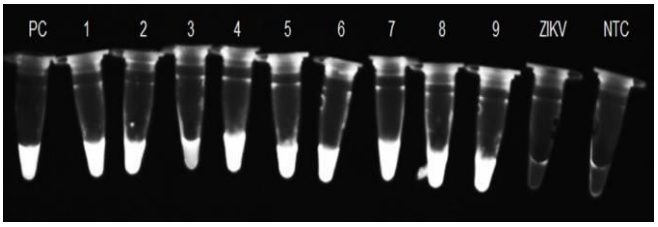

Figure 2: Visual observation of RT-LAMP assay products for detection of DENV in *Ae. aegypti* larvae pools with RNA extraction step. Amplification with nine DENV-specific primers on positive control with RNA of four DENV serotypes (Tube PC) and RNA isolation of larvae pools (Tubes 1-9); positive clinical samples to ZIKV (Tube ZIKV) and HCV (Tube HCV) were negative to DENV.

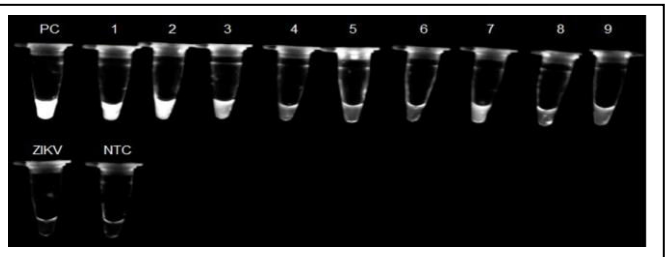

Figure 3: Visual observation of RT-LAMP assay products for detection of DENV in *Ae. aegypti* larvae pools without RNA extraction step. Amplification with nine DENV-specific primers on positive control with RNA of four DENVs serotypes (Tube PC) and samples of crude mosquito lysates (Tubes 1-9); positive clinical samples to ZIKV (Tube ZIKV) and HCV (Tube HCV) were negative to DENV.

LAMP assay is a promising tool for monitoring DENV in mosquitoes due to its high sensitivity and because it reduces equipment needs. On-site identification of infected mosquitoes with arbovirus will allow us to focus control efforts in sites of high epidemiological risk. Additionally, it will be able to carry out dengue and other arthropod borne disease surveillance in underdeveloped countries. Further studies in a greater number of samples in order to quantify viral detection through detection limit of RT-LAMP assay are ongoing.

CONCLUSION

In this research, four DENV serotypes were detected in immature stages of *Ae. aegypti* populations from Havana city by conventional RT-PCR assay such as it was previously reported in a study carried out in Arroyo Naranjo municipality from which three pools out of nine were positive to DENV-3 [6]. The above results showed RT-LAMP was able to detect DENV, with or without the RNA extraction stage, in a greater number of entomological samples than conventional RT-PCR without having cross-reactivity with closely related viruses [11,12,16,17], such as ZIKV and HCV. That reflects its application as a promising tool to dengue surveillance system in mosquitoes to prevent epidemic outbreaks in rudimentary laboratories or field locations of developing countries where RT-PCR resources are inaccessible due to minimal sample preparation and does not require expensive instrumentation and RNA isolation step.

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