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Dengue serotype detection from wild caught mosquito by reverse transcription loop-mediated isothermal amplification

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ABSTRACT *Dengue viruses are mainly transmitted by the Aedes aegypti mosquito which grows in breeding containers maintained by rain or human activity. The Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) reaction was found more sensitive and can detect 10-copies of RNA template whereas; real-time PCR (qPCR) had detection limits of 100 copies. To detect the dengue serotypes from wild-caught Aedes aegypti mosquitoes using a molecular technique RT-LAMP, and compare with qPCR to detect the same dengue serotype. This prospective analytical study was conducted at the Department of Virology, BSMMU, Dhaka, Bangladesh from January 2017 to December 2017. RT-LAMP was performed to detect dengue serotypes from wild-caught mosquitoes. Four different visual detection methods of RT-LAMP were conducted simultaneously. To confirm the test result, qPCR was done. Out of the 1348 wild-caught mosquitoes, 217(16.10%) were found positive for the dengue virus by RT-LAMP assay. Among this DEN-2 serotype was by far the highest percentage 184(84.79%) whereas, DEN-1 was 33(15.21%). The RT-LAMP is a relatively inexpensive, rapid, and simple tool for the accurate detection and serotyping of DENV from wild-caught mosquitoes.*

Keywords: Wild-caught Aedes aegypti mosquito, Loop-mediated isothermal amplification, qPCR.

Introduction

Dengue Fever (DF) or Dengue Haemorrhagic Fever (DHF) is caused by infection with any of the five Dengue virus serotypes (DV1-5) (Peters, 1997). Dengue viruses are mainly transmitted by the Aedes aegypti mosquito which grows up in breeding containers maintained by rain or human activity (Pan Am Health Organization, 1994; Bonilauri et. al., 2008 & Lu, 2009). In Bangladesh, particularly in Dhaka, epidemiological presentation of dengue such as fatal dengue hemorrhagic fever and dengue shock syndrome cases are changing rapidly and drastically (Dash, 2006; Vazeille, 2003). Between 2016 and 2017, two severe dengue outbreaks occurred in Bangladesh with a total number of 13,632 cases. It was found that Aedes aegypti has significantly more receptivity to DEN-1, DEN-2, and DEN-3 serotypes than other serotypes (Armstrong, 2003). In this study, the dengue serotypes detected in the female Aedes aegypti mosquito were from the

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wild caught mosquitoes collected from May 2016 to December 2017 during the dengue outbreak in Dhaka by using a molecular method, RT-LAMP. Mosquitoes were collected during the dengue outbreak from May 2016 to December 2017 for this study and stored at –80°C, but the detection of serotype in the lab by RT-LAMP was performed between January 2017 and December 2017.

RT-LAMP and qPCR on individual Aedes aegypti mosquito provided important findings of dengue serotype-specific vector infection rate within a particular geographical area (Fan, 1989; Ilkal, 1991; Victor, 2002). The RT-LAMP reaction was found more sensitive which can detect 10-copies of RNA template whereas; real-time PCR had detection limits of 100 copies (Sithiprasasna, 1994). RT-LAMP was previously performed by other authors to detect dengue viral serotypes from the blood samples of the dengue infected patients, however, for the first time it was performed to detect dengue viral serotypes from wild caught female Aedes aegypti mosquitoes which is completely a new approach. There was no known prior study that detects dengue viral serotype by RT-LAMP from mosquito.

Detection of dengue virus from wild caught mosquitoes was done by different methods such as Virus culture, qPCR or Immuno Chromatographic Test (ICT) by different authors earlier. However, all of these procedures have their potential drawbacks. While Virus culture and qPCR require more time, expense and expertise, and the specificity and the sensitivity of ICT is lower for the detection of viruses, RT-LAMP outweighs those disadvantages. The RT-LAMP has the potentials to serve as a relatively inexpensive, rapid, and simple tool for the early detection and serotyping of DENV from wild-caught mosquitoes.

This analytical study aimed to detect dengue serotypes from wild-caught Aedes aegypti mosquitoes using a molecular technique RT-LAMP and compare it with qPCR to detect the same dengue serotype. It will help to develop a sensitive, easy and inexpensive molecular detection technique at any laboratory setup worldwide.

Materials and Methods

This prospective analytical study was conducted from January 2017 to December 2017 for twelve months at the Department of Virology, BSMMU, Bangladesh. A total of field-collected wild 2,280 adult mosquitoes were brought to the Department of Virology, BSMMU between May 2016 and December 2017. The mosquitoes were sorted out as males and females, and eventually, 1,348 female Aedes aegypti were sorted out for RT-LAMP and were stored at –80°C. LAMP was performed on these mosquitoes to determine the dengue serotypes.

Virus Isolation and Detection

Individual mosquito were tested for the presence of the virus. The head of each mosquito was squashed by pressing it into an Eppendorf with a sterile wooden stick. Then 0.2mmol lysis buffer was added and kept at room temperature for two minutes to lyse the cell and make free dengue virus antigen. A separate PCR tube was used for each head spot. This technique was far more appropriate for virus isolation because it can easily detect 10-copies of RNA template; whereas, real-time PCR had detection limits of 100. Specificity and sensitivity of RT-LAMP is 10 times more than RT-PCR (Sithiprasasna, 1994).

Primer design

The RT-LAMP assay's serotype-specific oligonucleotide primers used for dengue viruses amplification were designed using the Primer-Explorer V3 software based on 3' non-coding region of (NCR) (GenBank accession number: 1) DENV-1 Western Pacific, U88535; 2) DENV-2 New Guinea C, AF038403; 3) DENV-3 H87, M93130, and 4) DEN-4 China Guangzhou B5, AF289029). Loop primers of it (Loop-F and Loop-B) were designed manually. The final primer sets of the group, specific for all of the four serotypes were as follows: DEN-F3 (forward outer primer), 5¢-GAGAAACCGCGTGTCAAC-3¢; DEN-B3 (the backward outer primer), 5¢-CCTTCCAATCTCTTTCCTGAA-3¢; DEN-FIP (the for-ward inner primer), 5¢ AGGGCCATGAACAGTTTTAATGGTCAGCTGACAAAGAGATTCTCA¢; DEN-BIP (the backward inner primer,) 5¢ CCTAACAATCCCACCAACAGCACCCTCTCAAAACATTGATAGC-3¢.

The RT-LAMP assay

The LAMP was performed with a loop-mediated RNA amplification kit (Eiken Chemical). A total of 25mL final reaction system composed of 2mL of template RNA, 1mL of Bst DNA polymerase mix, 1.6mmol L -1 each of inner primers FIP and BIP, 0.2mmol L -1 each of outer primers F3 and B3, and 1x Reaction Mix (Eiken Chemical). The final volume was adjusted to 25mL with distilled water. The reaction mixture was incubated at 63°C for 60 min in a loop amp real-time turbid meter (LA-320; Teramecs, Kyoto, Japan). The total run of PCR cycles was: 94°C for 4 minutes; 35 cycles at 94°C for 30 seconds, annealing at 55°C for 1 minute, extension at 72°C for 30 seconds. The reaction was terminated automatically by the inactivation of the polymerase at 80°C for 2 minutes.

Real-time Monitoring of Amplification by the RT-LAMP Assay and Endpoint Assessment

The real-time monitoring of the amplification of the dengue RT-LAMP assay was observed with a Loop amp real-time turbid meter (LA-320, Teramecs, Co., Ltd., Japan). The reaction was noted positive when the turbidity reached at 0.1 within 60 minutes at 650 nm. Additionally, 1mL SYBRGreen dye(Sigma-Aldrich, USA) was added into the tube to observe with the naked eye to notice the color change after the amplification procedure. A positive reaction was noted by a color change from violet to sky blue. Detection by ladder-like bands in agarose gel electrophoresis, 2% agarose gel was used. Amplified products were visualized by a gel documentation system by using UV Transilluminator (Wealtec Dolphin view, USA).

RT-LAMP's sensitivity was tested by 10 fold serial dilution of the samples. Specificity was tested by using mosquitoes carried chikungunya, confirmed by PCR. A negative control without any template was included in each test.

Statistical analysis: All data were analyzed by Statistical Package for the Social Sciences (SPSS) version 20, USA. Sensitivity, specificity, and positive and negative predictive values of RT-LAMP were determined using real time-PCR as the gold standard for diagnosis of dengue.

Mosquito Serotypes Detection by qPCR

qRT-PCR RNA from 140 µl of individual mosquito crushed samples was extracted by using the QIAamp viral RNA extraction kit (Qiagen Sciences, Valencia, CA, USA) as per the manufacturer's instruction. Sixty µl of elution buffer was used to elute the RNA and stored at -80°C. Ten µL of extracted RNA was used in all qPCR. In the case of mosquitoes, the entire mosquito was homogenized and the RNA was extracted from the homogenate using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocol. The RNA was re-suspended in 20 µl of RNAse free distilled water and stored at -80°C. A half µL of this RNA was used in the qRT-PCR. Using the reverse primer with AMV reverse transcriptase (Promega Corporation, Madison, WI, USA), the transcribed or viral RNA was reverse transcribed. The reverse transcription reaction was carried out at 42°C for 1 h. The cDNA thus obtained was used as the template in the qPCR. The TaqMan Universal PCR master mix (Applied Biosystems, Foster City, USA) was used in all qPCRs. Each reaction had 200 nM of forward primer, 250 nM of the probe, and 300 nM of reverse primer in a 25 µL final reaction volume. The PCR mixtures were pre-incubated at 50°C for 2 minutes followed by denaturation at 95°C for 10 minutes and 45 cycles of 95°C for 15 seconds and 60°C for 1 minute using the Applied Biosystems 7500 real-time PCR system. The final real-time data were analyzed by the SDS software, provided by Applied Biosystem.

Results

Among the total collected 2,280 wild-caught Aedes aegypti from different areas of Dhaka city 1,348(59.12%) were female Aedes aegypti. Out of these 1,348 mosquitoes, 217(16.10%) were found positive for dengue virus by RT-LAMP assay that was confirmed by another molecular technique qPCR.

Table-1 illustrates, among 217 positive reactions, 33(15.21%) were DEN-1 whereas; the most predominant serotype was DEN-2 with 184(84.79%). However, no DEN-3 or DEN-4 was detected from those mosquitoes. All the samples were run by qPCR for the serotype confirmation and found an equal result.

Table 1
Detection of Dengue Serotypes From Wild-caught Aedes Aegypti by RT-LAMP Assay and qPCR (n=217)

Serotypes of the study samples	Number of serotypes detected by RT-LAMP	Number of serotypes confirmed by qPCR
Dengue-I	33(15.21%)	33(15.21%)
Dengue-II	184(84.79%)	184(84.79%)
Dengue-III	00	00
Dengue-IV	00	00

Figure-1(a, b, c, d) depicts the detection of dengue viral serotypes from wild-caught mosquitoes by RT-LAMP's different visual methods; healthy control, negative control, DEN-1 positive, and DEN-2 positive have been marked in different images.

Positive amplifications showed step ladder patterns in gel electrophoresis, amplification curves in real-time, and color changes observed using HNB dye. The colur change expected for a Positive detection of the virus was blue.

Negative amplification (Healthy control, Negative control) showed no step ladder patterns in gel electrophoresis, no amplification curves in real-time; and no color change using HNB dye. The colur change expected for a Negative detection of the virus was purple.

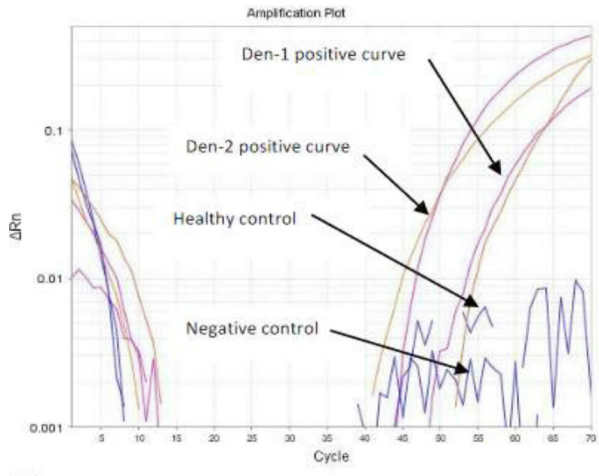

Figure 1. (a) RT-LAMP amplification curve

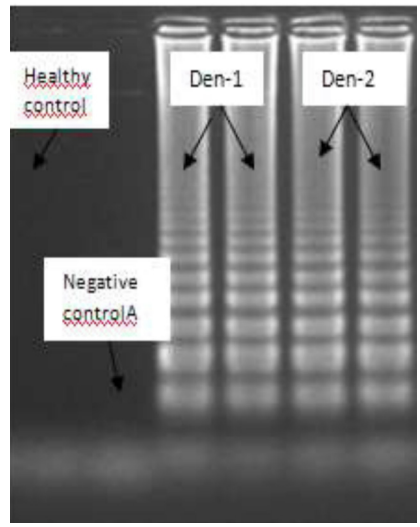

Figure 1. (b) Gel electrophoresis

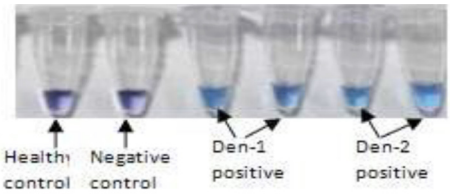

Figure 1. (c) Detection by HNB dye

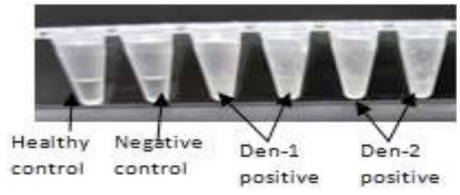

Figure 1. (d) Detection by turbidity

Four different visual detection methods after amplification [amplification curve in RT-LAMP - (1-a); ladder-like band in gel electrophoresis - (1-b); color change using HNB dye - (1-c); and turbidity - (1-d)] were performed for the detection of dengue serotypes from mosquito by RT-LAMP products in the study.

Discussion

Viral infections have become a serious public health concern nowadays (Shengfeng et al., 2009). The emergence or re-emergence of mosquito-borne viral diseases such as the DEN virus has been frequently reported worldwide and has caused a considerable economic loss for all (Lakshi et al., 2008;Tsetsarkin et al., 2011). It is now considered a real threat to hot temperate areas, such as Bangladesh, that is colonized by the Aedes mosquito (Coker, 2011; Parola et al., 2006; Karabatsos, 1975). The presence of infected mosquitoes in these localities is alarming and requires careful vector surveillance so that large outbreaks can be prevented.

In this study, out of 2,280 wild Aedes aegypti about 1,348(59.12%) were female Aedes aegypti. It may indicate that the female mosquito population is increasing than the male. However, to confirm, further extensive field investigation on mosquitoes is recommended.

After carrying out this study, we found that among the 1,348 female mosquitoes, 217(16.10%) were carrying the dengue virus in them. It indicates that even this percentage of the dengue virus vector can cause a severe dengue outbreak in a particular geographic location. Collection of the wild mosquitoes were done randomly during the dengue outbreak from May 2016 to December 2017, and from those randomly collected mosquitoes, about 16.10% were dengue mosquitoes which were detected by RT-LAMP and RT-PCR. It indicates that if there is around 16.10% dengue virus carrier mosquito in the environment, this may cause a severe dengue outbreak, and similar to what happened in Bangladesh in 2016's dengue outbreak (Mutsuddy, 2019).

Additionally, the detected DEN-2 serotype was by far the highest percentage, at 184(84.79%) whereas; DEN-1 was 33(15.21%). It means that the DEN-2 serotype is the commonly prevailing serotype in Dhaka. DEN-2 serotype is the serotype that causes more severe dengue haemorrhagic fever. If serotype is detected successfully, due measures could be taken to the patients which may reduce the dengue haemorrhagic fever and dengue haemorrhagic fever related mortality (Dash, 2006).

Relative sensitivity and specificity of RT-LAMP for wild-caught mosquitoes were 99% and 100% respectively. Four different visual detection methods that were conducted in this study, gave the same results after completion of amplification. This suggested that all types of detection methods had equal efficacy, specificity,

and sensitivity to detect the dengue virus. RT-LAMP's four detection methods (amplification curve, Gel electrophoresis, HNB dye and Turbidity) gave same results in detecting dengue serotypes. It indicated that all of the RT-LAMP's detection methods had same sensitivity and specificity in detecting dengue serotypes. Same sensitivity and specificity were found in the study by Lau et al. (2015).

Continuous surveillance of Aedes mosquito breeding and dengue infection is necessary for the prevention and control of dengue (Powers, 2000; Telles, 2009; Hyashi, 2007). To detect dengue serotypes from wild-caught mosquitoes RT-LAMP can be a stellar addition in any kind of laboratory setup. Portable RT-LAMP can be carried easily to the field level to detect dengue instantly. Colorimetric detection has made it much easier and has proved an immensely attractive feature to detect dengue serotypes from mosquitoes accurately.

Conclusion

Detection of dengue virus from mosquitoes can be done by different methods such as Virus culture, qPCR or Immuno Chromatographic Test (ICT). However, all of these procedures have their own limitations. While Virus culture and qPCR are more time-consuming, expensive and need expertise, and the specificity and sensitivity of ICT is lower in detecting viruses, RT-LAMP outweighs those disadvantages. The RT-LAMP has the potential to serve as a relatively inexpensive, rapid, and simple tool for the early detection and serotyping of DENV from wild-caught mosquitoes as well as from laboratory-reared infected mosquitoes. RT-LAMP method can be used in any kind of laboratory setup for DENV serotyping in both wild mosquitoes and laboratory-rear mosquitoes.

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