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Research Paper

Rapid detection of Yellow fever Virus with Recombinase Aided Amplification

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ABSTRACT

Here we report a rapid one-step recombinase aided amplification (RAA) assay for Yellow Fever Virus (YFV) detection. The YFV cDNA was first transcribed with the YFV RNA by a reverse transcriptase reaction, and the resulting product was then served as template for RAA amplification. The reaction was performed at a constant temperature of 39°C with a short detection time (less than 20 min). Our results indicated that the amplification is very sensitive, as low as 100 copies can be detected. The amplification is also specific as it had no cross reactions with other closely related viruses, such as Dengue virus, West Nile virus, Japanese encephalitis virus or Chikungunya virus. Hence, our method is of great potential in rapid detection and the control of YFV, and can be adapted to other applications.

Key words: Recombinase Aided Amplification (RAA), yellow fever virus.

INTRODUCTION

Yellow fever is an acute infectious, viral haemorrhagic disease caused by the causative agent Yellow Fever Virus that is transmitted by infected mosquitoes. Early symptoms of the yellow fever include sudden onset of fever, chills, severe headache, jaundice, bleeding, high fever, and eventually shock and failure of multiple organs (Onyango et al., 2004; Gardener and Ryman, 2010; van den Hurk et al., 2011; Quaresma et al., 2013). Worsening of the environment, rapid increase of tourism as well as decrease of the yellow fever virus vaccination coverage may be the major factors that accounted for a constant rise of yellow fever cases, particularly in Africa. As WHO reported that there are estimated 200, 000 cases of yellow fever, causing 30, 000 deaths, worldwide each year, with 90% occurring in Africa (Beasley et al., 2015). The Yellow fever disease remains to be a major threat to the public health.

YFV belongs to the Flaviviridae family (Chambers et al., 1990), and is a 40-60 nm wide-enveloped, icosahedral nucleocapsid RNA virus. The positive-sense, single-stranded RNA is around 11,000 nucleotides long and has a single open reading frame encoding a polyprotein, and it also has a short 5' UTR of 118 nucleotides, 3' UTR of 511 nucleotides, a 5' cap structure (me7-GpppA-me2) (Brendenbeek et al., 2003). This genus also includes the West Nile virus, Dengue virus, Zika virus, Japanese encephalitisvirus and several other viruses (Gould and Solomon, 2008).

This disease may well be spreading to new areas as the yellow fever cases are still on the rise. Though, currently its widespread in China has not been the case, an unexpected outbreak is possible as the climate conditions are very similar to those in Africa in some areas of southern part of China (Shi et al., 2009). Meanwhile, modern transportation facilities have provided a convenient tool for people to move frequently around the world, which also favors a widespread of the YFV and its carriers (Carrington and Auguste, 2013; Lü et al., 2010). Therefore, developing a method for rapid and accurate detection of the yellow virus for effective prevention and control of the disease is vital.

Taking the advantage of the RAA (Lü et al., 2010; Camille et al., 2013), here we report a one-step amplification method to detect the YFV by RT-RAA. In the process, the YFV

cDNA was first obtained by reverse transcriptase reaction, and the resulting cDNA was then served as template for amplification by the RAA at 39°C, and specific detection of the YFV was achieved within 20 min.

MATERIALS AND METHODS

Viral RNA

The RNA of YFV was isolated from Yellow fever vaccine (live sample) which were purchased from Beijing Tiantan Biological Products Co, Ltd; the RNA of Japanese encephalitis virus was isolated from Japanese encephalitis vaccine (live sample) which was purchased from Chengdu institute of Biological Products Co, Ltd; the dengue fever virus, Chikungunya virus and West Nile virus plasmid samples were provided by Zhejiang International Travel Healthcare Center.

Reagents and the detection equipment

RNA isolation kit was purchased from life technologies (Ambion1836, USA). M-MLV reverse transcriptase was purchased from Promega (Cat.M1701).

RAA kit and the detection equipment (QT-RAA-F610) were provided by Jiangsu Qitian Gene Biotech Co., Limited, China). Specific primers and probes for detection of the corresponding viruses were synthesized by Shanghai Bio-engineer Co., Limited, China).

RNA isolation

RNA of the target viruses was isolated by magnetic bead-based purification method according to the manufacturer's instructions.

Design and synthesis of the RAA primers and probes

The genome sequence of the yellow fever virus was obtained from the NCBI database (USA). The specific primer and probe sequences were designed using the DNAMAN7.0 or Vector NTI 11 softwares.

The establishment of amplification system for the yellow fever virus using the RAA kit

The RAA reaction was carried out in a thin 0.2 ml tube containing appropriate concentration of the RAA enzyme mix in the lyophilized forms (Lü et al., 2010). All other components (Table 1) were added and mixed well before the magnesium acetate was added to initiate the reaction as described previously (Lü et al., 2010), and the tube was then transferred to the detection equipment for specific amplification at 39°C for 20 min. The RAA-Flow device was connected to a computer so that the real-time fluorescence signals can be captured and monitored. A control experiment was done side by side, and the control reaction contained all other components except for the template DNA.

For optimized amplification efficiency, a series of dilutions of the M-MLV reverse transcriptase was tested in our experiments, and the best results were obtained when 40 units of the enzyme was added in the reaction.

Sensitivity test using different dilution of the template DNA

A fragment of the yellow fever virus was synthesized, which was then subcloned into a plasmid vector so that the amplification sensitivity in the reaction could be easily monitored. A dilution series was prepared, and each contained a different copy number per micro liter (µL) of the yellow fever virus DNA (10^7, 10^6, 10^5, 10^4, $10^3$and 10^2, respectively), which was then used to test the detection sensitivity in the RAA detection system as above so that an optimized concentration threshold was obtained for further detection assay.

To test the sensitivity of the detection, a dilution series of a plasmid containing a specific yellow fever virus fragment was used in the detection reaction, and specific amplification could be still obtained when the plasmid DNA concentration was at 100 copies.

Specificity comparison tests

To test the detection specificity, and also to eliminate cross reactions from the members in the Flaviviridae family, Dengue, West Nile, Zika, Japanese encephalitis, Chikungunya and several other viruses were used for detection experiments as described above.

For cross reaction check, West Nile, Dengue, Chikungunya as well as Japanese encephalitis viruses were also tested, and no cross reactions were detected.

RESULTS AND DISCUSSION

The yellow fever virus genome information was obtained from the NCBI database, and the sequence IDs used for designing primers and probe in this report included AY640589.1, JX898871, JX898876, JX898869, JX898870, JX898873, AY603338.1 and AF094612.1. The specific primer set and probe were designed using the Vector NTI (Invitrogen) software and are shown in Table 2. We chose to use the sequences of the primer set and probe that had

Table 1. The RAA detection system.

Total Volume	**50 µL**
ddH_2O	16.7 µL
2 x buffer	25 µL
Forward Primer (10 µM)	2.1 µL
Reverse Primer (10 µM)	2.1 µL
Probe (10 µM)	0.6 µL
M-MLV reverse transcriptase	20-100 units
Yellow fever virus Template	1 µL
Magnesium acetate (280 mM)	2.5 µL

Note: the lyophilized forms of the RAA enzymes were not listed here.

Table 2. Specific primers and probe used in the paper.

primer/probe	**DNA sequences (5'-3')**	**Anticipated fragment**
Forward primer	AAATCCTGKGTGCTAATTGAGGTGYATTGG	119 bp
Reverse primer	ACATDWTCTGGTCARTTCTCTGCTAATCGC	
Probe	GCAAATCGAGTTGCTAGGCAATAAACACATT(FAM-dT)G(dSpacer)A(BHQ1-d)TAATTTTRATCGTTC(phosphate)	

been reported to work with high specificity and efficiency (Camille et al., 2013) for our experiments so that a direct comparison of the amplification specificity and sensitivity could be carried out more easily.

As we know, specific amplification of the DNA in PCR depends on the amount of template DNA. In our one-step amplification experiments, the amount of the DNA template is also very much dependent of the reverse transcriptase added to the reaction system. Hence, different concentrations of the reverse transcriptase (different units, 20 U, 40 U, 60 U and 100 U, respectively) were added to the reaction system for testing, respectively. Our results indicated that the reaction with 40 U of the reverse transcriptase gave the best specific amplification in less than 20 min (Figure 1). All other amplification reactions herein were carried out under these optimized reaction conditions.

We synthesized a fragment of the yellow fever virus based on the genome information, and this fragment was subcloned into a plasmid vector so that the amplification sensitivity in the reaction could be easily monitored. As seen in Figure 2, different copy numbers of the plasmid DNA resulted in different amplification patterns. We also noticed that most amplifications could be achieved under 20 min, however, the specific amplification time extended as the template DNA copy numbers added in the reactions decreased, and 100 copies of the DNA could still be sufficient for specific amplification.

As the members of the Flaviviridae family may contribute to cross reactions and result in nonspecific amplification, and so we conducted a comparison test for specificity using different templates from Dengue, West Nile, Zika, Japanese encephalitis, Chikungunya viruses. Our results indicated that no cross reactions was observed, and rather a specific amplification for the yellow fever virus was clearly evident (Figure 3).

Detection of the YFV in the laboratory is usually done with serological test, or by identification and isolation of the virus culture or with other molecular biological methods. Generally, detection by identification and isolation culture of the virus from the infected animals is more reliable. However, this is always time-consuming, laborious. On the other hand, the commonly used serological test required relatively a large amount of the virus for a positive detection (Lü et al., 2011). Compared to the above detection approaches, PCR based detection is more sensitive, more specific amplification can be achieved in a much shorter period of time. PCR based procedures have been widely applied to the field of the microbiological detection, and can detect the virus more easily, and also require much less materials for detection (Gibney et al., 2012). These included several basic PCR methods, reverse transcriptase PCR, real-time fluorescent PCR or nested PCR methods (Terpe, 2013; Méndez et al., 2013). Though these methods worked reliably, they all depend on accurate laboratory settings and require more expensive equipment (Dash et al., 2012; Bae et al., 2003; Mantel et al., 2008; Weidmann et al., 2010; Nunes et al., 2011). Therefore, in response to an outbreak like yellow fever virus, a portable device with simple and yet reliable

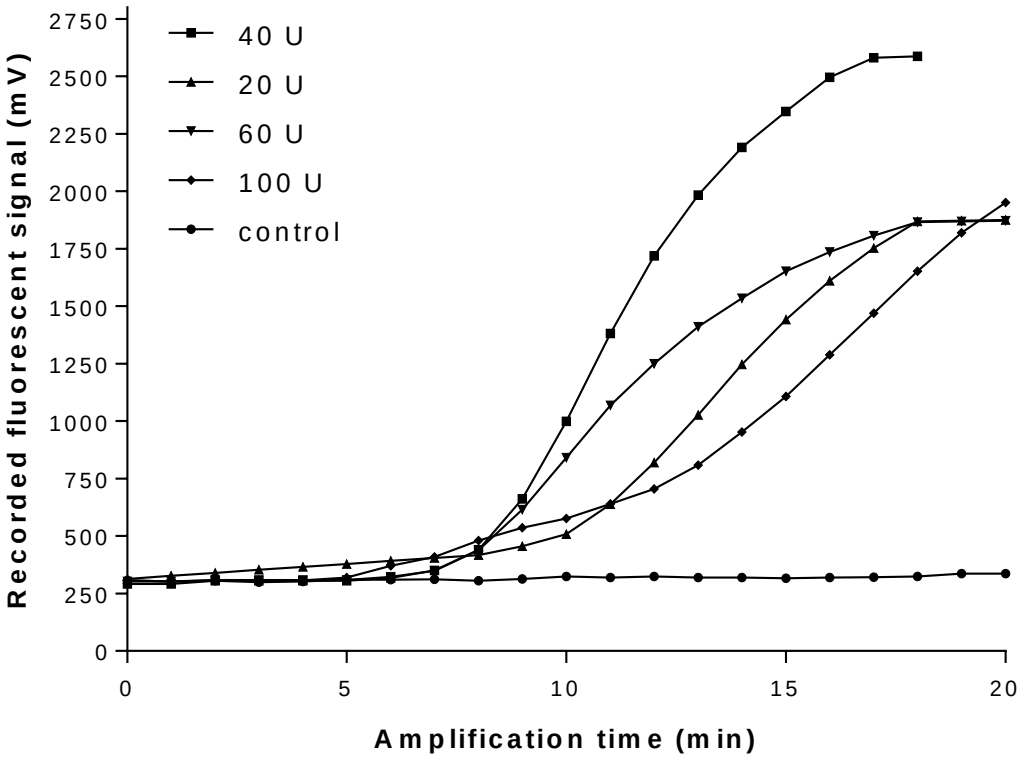

Figure 1. Specific detection of the Yellow Fever Virus by RT-RAA amplification under different M-MLV enzyme concentrations.

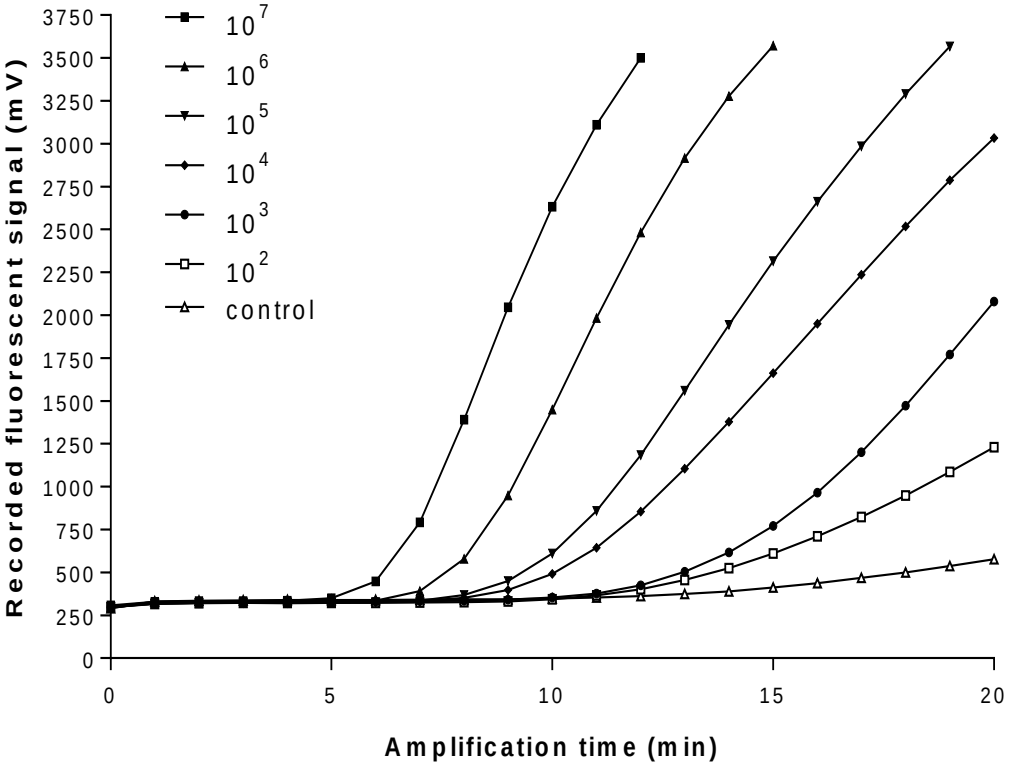

Figure 2. Specific amplification with different copy numbers of the plasmid DNA in the reaction.

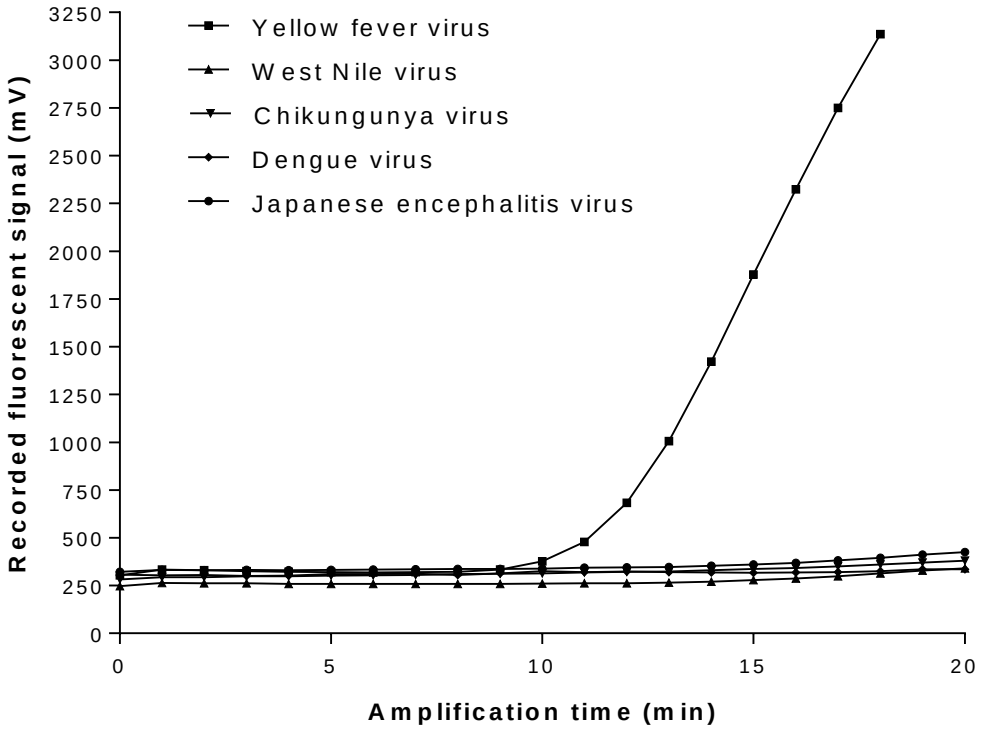

Figure 3. Amplification patterns using different templates from several members of the Flaviviridae family.

assay of the virus and with relatively low cost is much better suited for rapid and on-site detection. As reported elsewhere, RAA offers rapid specific amplification under low and constant temperature, and the reaction can be achieved in between 15 and 30 min under normal room temperature and results in rapid, sensitive and specific detection of the target.

In this report, we established a RT-RAA based fluorescent procedure for the yellow fever virus detection, which could readily detect the virus at 39°C in 20 min (in essence, a detection signal could be noticed in 8 min). We have proved that this amplification was specific and sensitive, and the lowest threshold of 100 copies of the plasmid DNA could still yield a specific amplification. No cross reactions from the other members of the Flaviviridae family was observed. In summary, our RT-RAA based method for the yellow fever virus detection offers greater advantages over the other PCR-based methods and will be of greater prospect, and can be adapted easily for high throughput detection for other biological sample detections.

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