International Journal of Infectious Diseases 78 (2019) 93–98

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Rapid detection of Banna virus by reverse transcription-loop -mediated isothermal amplification (RT-LAMP)

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ARTICLE INFO

Article history:
Received 21 September 2018
Received in revised form 29 October 2018
Accepted 29 October 2018
Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Banna virus
RT-LAMP
Mosquito
Detection

ABSTRACT

Objectives: Banna virus (BAV) is classified in the genus *Seadornavirus* within the *Reoviridae* family and considered to be an emerging pathogen. We aimed to develop a rapid and simple molecular detection approach for all BAV subgroups in isothermal conditions.
Method: A set of six specific primers was designed to target the segment 12 of BAV, and the reverse transcription-loop mediated isothermal amplification (RT-LAMP) assay was developed and compared with conventional RT-PCR method.
Results: The amplification of the RT-LAMP assay can be obtained within 40 min at 65 °C. The results from specificity showed that only target BAVs RNA including genotypes A, B and C were amplified and the assay demonstrated a sensitivity of 3.6×10^{-2} PFU/mL, which was higher than conventional RT-PCR measurement. A good reliability for the assay was presented in the further evaluation for BAVs RNA from serial diluted BAV-spiked serum and 47 pools of field mosquito samples.
Conclusions: Our findings present a rapid, sensitive and specific RT-LAMP assay that can be applied for BAV detection in clinical or field samples in the future.

Introduction

Banna virus (BAV) is classified in the genus *Seadornavirus* within the *Reoviridae* family. BAV was first isolated in 1987 from cerebrospinal fluid and the sera of human patients with encephalitis in Yunnan, China (Attoui et al., 2005). Subsequently, the virus has been isolated from a diverse group of vertebrates and invertebrates, including mosquitos, ticks, midges, cattle, and pigs from different regions in China, Vietnam, and Indonesia (Nabeshima et al., 2008; Liu et al., 2010, 2016b). BAV is considered to be an emerging pathogen that can result in human infections with possible manifestation of fever and viral encephalitis. There is growing evidence showing that the geographic distribution of BAV is expanding, and more surveillance of BAV is required (Reuter et al., 2013; Liu et al., 2016b).

BAV is a double-stranded RNA virus that is composed of 12 segments within its genome. The genome size is approximately 21 kb, and the length of the segments range from 862 (segment 12) to 3,747 bp (segment 1). Segments 1, 2, 3, 4, 8, 9, and 10 encode the seven structural proteins (VP1, VP2, VP3, VP4, VP8, VP9, and VP10), respectively. Segment 7 encodes a protein kinase, while segment 12 encodes a dsRNA-binding protein (Attoui et al., 2000; Jaafar et al., 2005). The remaining segments 5, 6, and 10 encode nonstructural proteins with unknown function. Segment 12 is a highly conserved region within the BAV genome, and the available sequence information for this segment is the most abundant in the NCBI database (Liu et al., 2016a). Phylogenetic analyses of BAV based on the complete coding sequence of segment 12 indicate that the virus can be classified into 3 groups, A, B and C. Isolates from China and Vietnam are clustered in group A, strains from Indonesia are classified in group B, and the strains from *Anopheles sinensis* mosquitoes and Odonata from central China formed a newly clustered group C (Liu et al., 2016a; Xia et al., 2018a).

Reverse transcription-loop mediated isothermal amplification (RT-LAMP) is a nucleic acid amplification approach that amplifies reverse transcribed DNA from RNA using strand displacement DNA polymerase under isothermal conditions (Notomi et al., 2000). Due to its rapidness, simplicity, sensitivity and specificity, RT-LAMP has been successfully applied in the detection of various RNA viruses

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https://doi.org/10.1016/j.ijid.2018.10.025

(Parida et al., 2005; Chen et al., 2011; Li et al., 2011; Hindupur et al., 2016; Oloniniyi et al., 2017).

In this study, we describe a rapid, sensitive and specific RT-LAMP detection assay for BAV that targets segment 12. This technique might be helpful for the screening or surveillance of BAV in field or clinical samples in future epidemiological studies.

Material and methods

Cells and viruses

C6/36 cells (*Aedes albopictus*) were maintained at 28 °C in RPMI 1640 (Gibco, USA) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). BAV strains YN15-126-01 and HB14-71-01 were isolated from field-caught mosquitos and preserved in our laboratory (Xia et al., 2018a). The virus was propagated in C6/36 cells, and cultured supernatants were harvested at four days post-infection, clarified through centrifugation at $5000 \times g$ for 10 min and then stored at −80 °C until use.

The virus infectious titer was determined by the plaque assay. C6/36 (2.5×10^5) cells in 500 μL RPMI 1640 were seeded in each well of a 24-well plate. Ten-fold serial dilutions of the different viral suspensions were added to the wells and incubated for 1 h at 28 °C. Thereafter, the virus supernatant was replaced with RPMI 1640 containing 2% FBS, 1% penicillin/streptomycin and 1% methylcellulose for 72 h incubation. After the culture medium was removed and a 2 h fixation step with 3.7% formalin in PBS was implemented, the plaques were read by being stained with 1% crystal violet. Counts were expressed as plaque-forming units per milliliter (PFU/mL).

Virus-spiked serum and field collected mosquito samples

Normal human serum (Sigma-Aldrich, USA) samples were spiked with BAV strain YN15-126-01. Exactly 100 μL of BAV at 1.44×10^3 PFU/mL was mixed with 300 μL normal serum to obtain spiked samples with titers of 360 PFU/mL. Then, samples were serially diluted four-fold to obtain spiked samples with titers containing 90, 22.5, 5.6, 1.4, and 0.35 PFU/mL.

A total of 47 pools of mosquitos (50 mosquitos/pool) including *Aedes albopictus*, *Anopheles sinensis*, *Culex quinquefasciatus* and *Culex tritaeniorhynchus* were collected from Hubei, Yunnan, and Guagnzhou during 2014, 2015 and 2017. The pooled mosquitos were homogenized, further clarified by centrifugation and then filtered through a sterile 0.22 μm filter (Millipore, Billerica, USA) to remove bacteria and other debris.

RNA extraction

RNA was extracted from 140 μL of BAV-infected C6/36 cell culture supernatant, BAV-spiked human serum or filtered mosquito homogenate samples from the above steps using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The RNA was eluted in 60 μL of elution buffer and stored at −80 °C until use.

Preparation of artificial RNA

The complete sequences of segment 12 from BAV strains 02VN018b, BJ95-75, GS07KD30, JKT-6423, and SX0790 were synthesized and cloned into the plasmid vector pBlunt with a T7 promoter (Wuhan Tianyi Huiyuan Bioscience & Technology Inc). The complete genome sequence of dengue virus 2 (DENV-2, strain TSV01) was constructed, preserved by our group and cloned into pBlunt. The complete genome sequences of Chikungunya virus (CHIKV, strain CHIK-SY), Japanese encephalitis virus (JEV, strain SA14), West Nile Virus (WNV, strain3356K), yellow fever virus (YFV, strain 17D), and Zika virus (ZIKV, strain SZ_WIV01_ZB) were cloned into a plasmid vector under the expression of the T7 promoter (provided by Prof. Bo Zhang's laboratory in the Wuhan Institute of Virology, Chinese Academy of Sciences).

The above listed plasmids were linearized by restriction enzyme digestion as a template for *in vitro* RNA transcription using the HiScribe™ T7 Quick High Yield RNA Synthesis Kit (NEB, Ipswich, USA) according to the manufacturer's instructions. The transcripts were purified using the RNeasy Plus Mini kit (Qiagen, Hilden, Germany) and resuspended in 40 μL of RNase-free water. The concentrations of *in vitro* transcribed RNAs were determined using a NanoDrop ND-2000 (Invitrogen, Carlsbad, USA).

Primer design

Prior to designing the specific primers, a total of 38 available sequences of segment 12 for BAV [China (n = 33), Vietnam (n = 2), and Indonesia (n = 3)] (Supplementary Table 1) covering were retrieved from GenBank. The sequences were aligned and further analyzed using Bioedit v7.0.5 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and conserved regions were identified as targets for LAMP primers. A specific set of RT-LAMP primers was designed to amplify the conserved target fragments of segment 12 using PrimerExplorer V5 (Eiken Chemical Co., Ltd., Japan; http://primerexplorer.jp/e/). For verification of specificity, the designed primers were queried on the NCBI BLAST tool with use of the nucleotide database as the reference. The primers were synthesized and mixed together based on the recommended concentrations provided by Wuhan Tianyi Huiyuan Bioscience & Technology Inc.

RT-LAMP assay

In running the RT-LAMP assay, a DEAOU RNA Amplification Kit (RT-LAMP) (DEAOU, Guangzhou, China) was used. Final amplification was carried out in a total volume of 25 μL consisting of the following reagents (final concentration): $2 \times$ RM 12.5 μL, 0.8 μL Bst

Table 1
Sequences and concentrations of BAV RT-LAMP primers.

Primer name	Position[a]	Sequence (5′ to 3′)	Concentration[b] (μM)
F3	638–657	AAACAGCTGCAGATTCAGGT	0.2
B3	820–839	CGTGCTATTCGGGATGAAGA	0.2
FIP (F1c + F2)	707–727, 660–679	ACGGCACAGGACCAGTAGTCT-CCGCTTAAGTAGCCATGGAG	1.6
BIP (B1c + B2)	738–758, 800–817	GTTGGGACTAGGTCGCCATGC-ATAGTGGAAGGGTGTGGGA	1.6
LF	681–703	GGTGGTCCATAAAATTACCGACG	0.8
LB	769–793	ACTAGGATGTAAGGTTTAGATCGGA	0.8

[a] Primer position in BAV strain JKT-6423 (accession number: NC_004198).
[b] The concentration is the final concentration in the reaction mix.

DNA polymerase, 0.5 μL SYBR Green I, and 0.2 μL Reverse transcriptase. The amount of primer needed for one reaction was 16 μM pmol for FIP and BIP, 0.2 μM for F3 and B3, and 8 μM LF and LB. In the final step, 2 μL of target RNA was added to the reaction tube. The reactions were conducted under isothermal temperature of 65 °C for 40 min using an Automated Isothermal Amplification and Detection System (Shanghai Cohere electronic technology Co., LtD., Shanghai, China).

One step Reverse Transcript- PCR (RT-PCR) amplification

One step RT-PCR amplification for BAV was performed using Prime Script™ One Step RT-PCR Kit Ver.2 (Takara, Dalian, China). The PCR primer set (12-B2-S: CAGAGTATAAATCAATCGCCCAAG; 12-B2-R: GTTCTAAATTGGATACGGCGTGC) used in BAV amplification was adapted from a previously reported study (Billoir et al., 1999). The final reaction mixture (total volume, 25 μL) contained 12.5 μL of 2 × 1 Step Buffer, 1.0 μL Prime Script 1 Step enzyme Mix, 1.0 μL each of primers (10 μM), 7.5 μL H_2O, and 2.0 μL RNA samples. Thermal cycling was initiated at 50 °C for 30 min, 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. PCR products were visualized in a 1.5% agarose gel stained with ethidium bromide.

Specificity and sensitivity of RT-LAMP detection

The viral RNA samples that originated from BAV strains YN15-126-01 and HB14-71-01; artificial RNAs of BAV strains 02VN018b, BJ95-75, GS07KD30, JKT-6423, and SX0790; and other representative mosquito-borne virus samples such as DENV-2, JEV, YFV, CHIKV, WNV, and ZIKV were used to detect the specificity and compatibility of the RT-LAMP assay.

To test the detection limit of the RT-LAMP assay for BAV, a standard sample obtained from C6/36 cells infected with BAV strain YN15-126-01 was used at a concentration of 3.6×10^5 PFU/mL. The standard sample was serially diluted 10-fold from 3.6×10^4 to 3.6×10^{-3} PFU/mL to estimate the limit of viral particles detected. The viral particle detection limit of the RT-

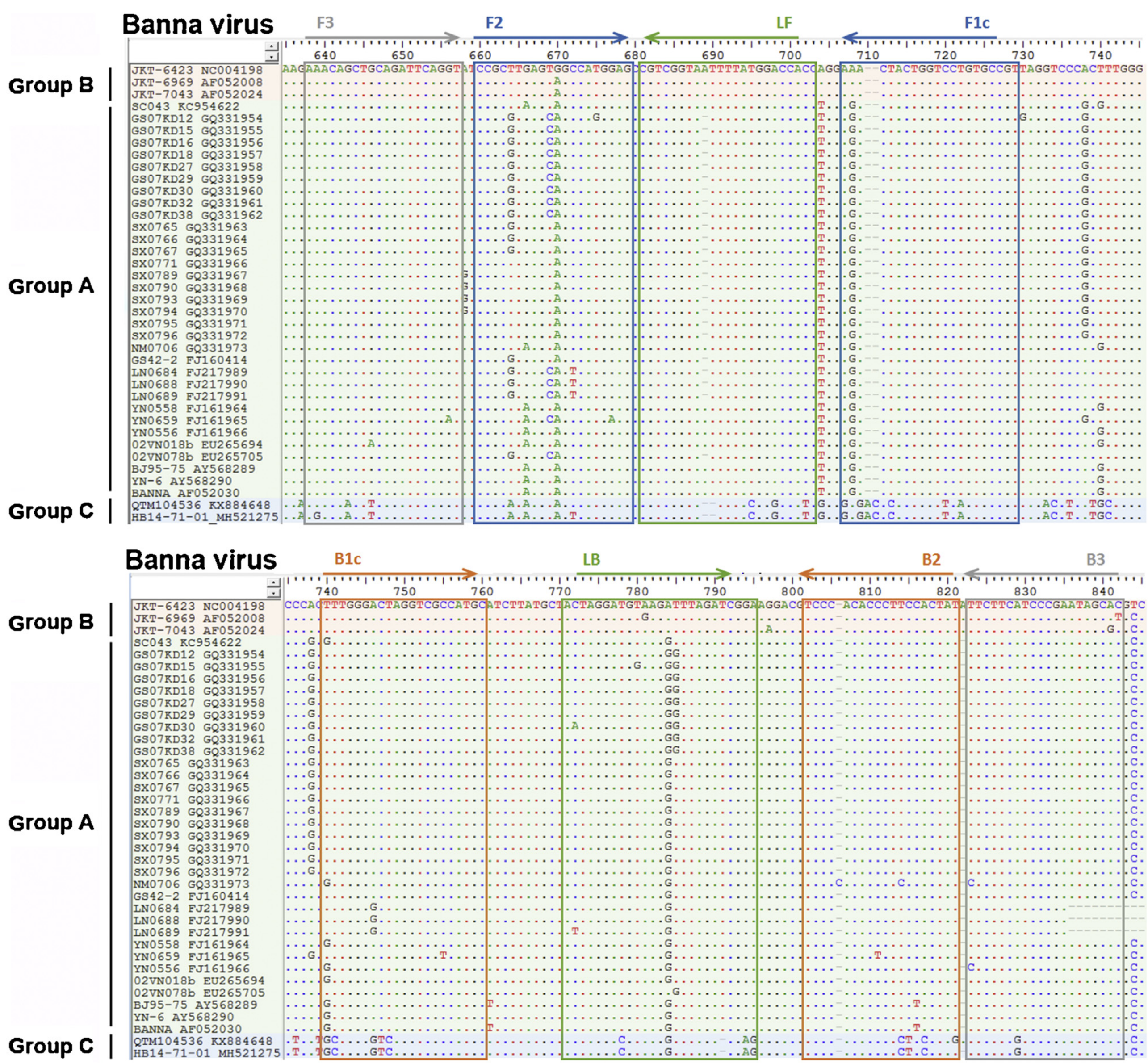

Figure 1. Alignment of BAV sequences and positions of RT-LAMP primers. Boxes represent the sites recognized by each oligonucleotide primer, and arrows show the direction of each primer. The name and accession numbers for the BAV strains were shown on the left.

LAMP assay was compared with that of a conventional RT-PCR method, and the tests were repeated 3 times to demonstrate reproducibility.

Detection of BAV from spiked serum and field mosquito

RNAs for the normal human serum, BAV-spiked serum (with titers of 360, 90, 22.5, 5.6, 1.4, and 0.35 PFU/mL), and mosquito pools from Hubei (15 pools), Yunnan (17 pools), and Guangzhou (15 pools) were detected by the RT-LAMP and conventional RT-PCR assays concurrently, and the comparison was repeated 3 times.

Results

RT-LAMP primer for BAV

We designed BAV RT-LAMP primers that targeted conserved sequences in segment 12, which consisted of a set of six primers: two outer primers (F3 and B3), a forward inner primer (FIP: F1c + F2), a reverse inner primer (BIP: B1c +B2), a forward loop primer (LF), and a reverse loop primer (LB). The sequences, locations, and concentrations of the oligonucleotide primers are shown in Table 1. The designed primers were evaluated based on the sequence alignment of segment 12 from 38 available BAV strains in GenBank as of October 2018. Twenty-three sequences of group A1 isolates, ten of group A2, three of group B, and two of group C were used (Supplementary Table 1). The designed RT-LAMP primers contain 169 nucleotides in total length and recognize eight distinct sites in segment 12 of BAV (Figure 1). The primers had 147 out of 169 (87.0%), 165 out of 169 (97.6%), and 138 out of 169 (81.7%) identical residues to the segment 12 of group A, B, and C BAV sequences, respectively; this outcome indicated sufficient specificity for use with all of the three groups of BAV.

Specificity of RT-LAMP detection

The specificity of RT-LAMP detection was determined with RNA samples of groups A and B BAVs, DENV-2, JEV, YFV, CHIKV, WNV, and ZIKV. All of the BAV samples (genotypes A, B, and C), which were isolated from China, Vietnam, and Indonesia, gave a positive RT-LAMP reaction in approximately 10–20 min, while no rising curve of fluorescence was observed with the samples of DENV-2, JEV, YFV, CHIKV, WNV, or ZIKV (Figure 2A). In addition to observations by real-time fluorescence, we also used visual inspection to determine a positive reaction after the reaction was terminated at 40 min. The results of the BAVs yielded a positive color change to green, whereas the samples of DENV-2, JEV, YFV, CHIKV, WNV, or ZIKV displayed a light-orange color, similar to the negative control (Figure 2B).

Sensitivity of the RT-LAMP assay

The detection limit of the RT-LAMP assay monitored by real-time measurement of fluorescence was approximately 10^{-2} PFU/mL (Figure 3A). The detection limit of the RT-LAMP assay by direct visual detection was also 10^{-2} PFU/mL. By comparison, the limit of conventional RT-PCR indicated that the limit of detection was approximately 10^{0} PFU/mL (Figure 3B), 100 times higher than that of the RT-LAMP assay.

Detection of BAV in BAV-spiked serum and field mosquito samples

Due to the current non-availability of a BAV-positive human blood sample, the feasibility of using the RT-LAMP assay for clinical specimens was evaluated using BAV-spiked human serum samples. Using the RT-LAMP assay, we detected viral RNA in serum at a titer of 1.4 PFU/mL in triplicate reactions (Table 2), and the time to obtain a positive result (Tp) was 34 ± 1.5 min. However, for conventional RT-PCR, there was only one positive in triplicate serum assays for 1.4 PFU/mL spiked samples, indicating that conventional RT-PCR was less sensitive than RT-LAMP in spiked serum samples. Six out of 23 mosquito samples from Hubei and Yunnan were positive for BAV by RT-LAMP detection. However, all mosquito samples collected in Guangzhou in 2017 were negative. These results were concordant with those of conventional RT-PCR assay (Table 3), and the RT-LAMP assay did not show any false-positive results.

Discussion

Recent studies for BAV indicated that the viruses are currently undergoing rapid evolution (mean nucleotide substitution rate was estimated at 2.46×10^{-2} substitutions per site per year) and the geographic area for the BAV habitat is expanding (Reuter et al., 2013; Liu et al., 2016b), which indicates that surveillance for BAV is required. Since the most common symptom of BAV infection is fever with non-specific clinical features when compared to the other mosquito-borne diseases, laboratory diagnostic methods are required for screening or identification (Attoui et al., 2005; Tao and

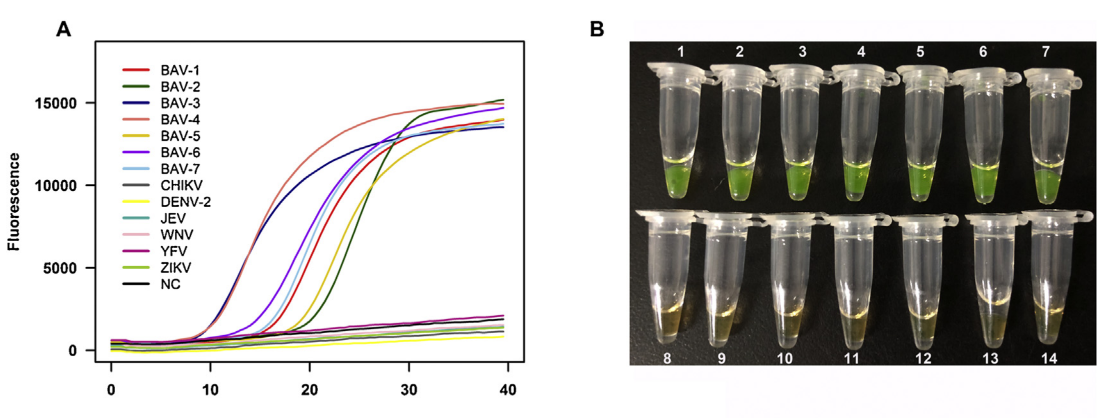

Figure 2. Specificity and compatibility of RT-LAMP measurement for the detection of BAV. (A) RT-LAMP assays were monitored by real-time measurement of fluorescence within 40 min. Positive reactions were only observed in the BAV viruses. BAV-1 to BAV-7, strains YN15-126-01, HB14-71-01, JKT-6423, 02VN018b, BJ95-75, SX0790, and GS07KD30; (B) Visual detection of RT-LAMP assay. The tubes represent BAV strains and the negative controls used in the visual inspection. 1-7, BAV strains YN15-126-01, HB14-71-01, JKT-6423, 02VN018b, BJ95-75, SX0790, and GS07KD30; 8, CHIKV; 9, DENV-2; 10, JEV; 11, WNV; 12, YFV; 13, ZIKV; 14, Negative control (NC).

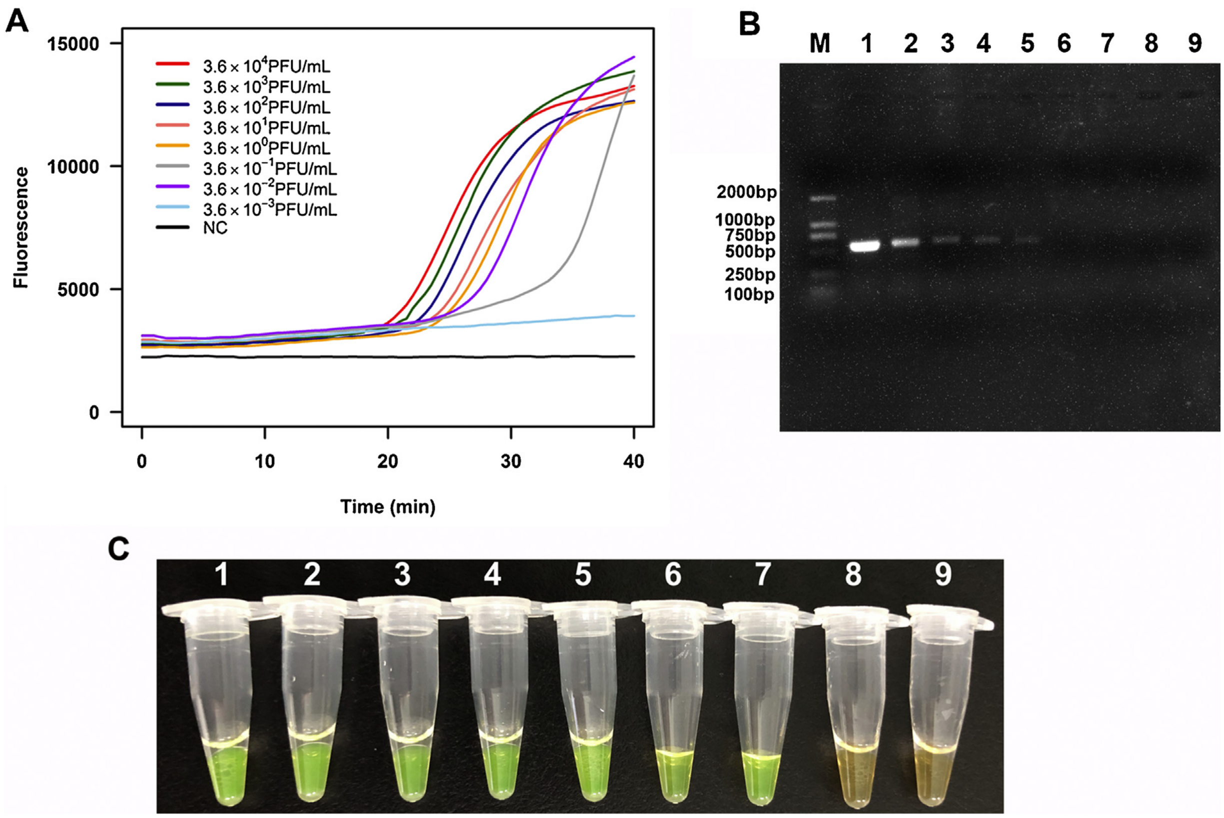

Figure 3. Sensitivity of RT-LAMP detection for BAV. (A) Fluorescence for the RT-LAMP assay was monitored by real-time measurement of fluorescence within 40 min; (B) The PCR products revealing a 500-bp specific amplicon for BAV were analyzed by 1.5% agarose gel electrophoresis and strained with ethidium bromide. Lane M, DNA marker; 1, 3.6×10^{4} PFU/mL; 2, 3.6×10^{3} PFU/mL; 3, 3.6×10^{2} PFU/mL; 4, 3.6×10^{1} PFU/mL; 5, 3.6×10^{0} PFU/mL; 6, 3.6×10^{-1} PFU/mL; 7, 3.6×10^{-2} PFU/mL; 8, 3.6×10^{-3} PFU/mL; 9, Negative control; (C) Visual detection of RT-LAMP assay. The tubes represent samples used in the visual inspection of RT-LAMP assay. 1, 3.6×10^{4} PFU/mL; 2, 3.6×10^{3} PFU/mL; 3, 3.6×10^{2} PFU/mL; 4, 3.6×10^{1} PFU/mL; 5, 3.6×10^{0} PFU/mL; 6, 3.6×10^{-1} PFU/mL; 7, 3.6×10^{-2} PFU/mL; 8, 3.6×10^{-3} PFU/mL; 9, Negative control (NC).

Table 2
Detection of BAV in virus-spiked serum samples.

Sample	PFU/mL	RT-LAMP		Conventional RT-PCR
Serum		Positive	Tp[a] (min)	Positive
	360	3/3	19 ± 1.6	3/3
	90	3/3	26 ± 0.6	3/3
	22.5	3/3	27 ± 0.3	3/3
	5.6	3/3	30 ± 0.5	3/3
	1.4	3/3	34 ± 1.5	1/3
	0.35	1/3	–	0/3
Mock	0	0/3	–	0/3

[a] Tp: Time to obtain positive results.

Chen, 2005). This is the first time the development of an RT-LAMP detection assay for BAV has been reported.

The detection limit of the RT-LAMP assay for BAV could reach 3.6×10^{-2} PFU/mL for cell-infected supernatant and 1.4 PFU/mL for spiked serum, yielding 10-fold higher sensitivity than that for conventional RT-PCR. Furthermore, RT-LAMP primers can specifically recognize a target sequence from 8 independent target sequence regions, in comparison with PCR primers that can recognize only two independent regions. Our result shows that the assay could detect representative BAV isolates either from group A, B or C with no cross-reactivity with other tested mosquito-borne viruses such as DENV, CHIKV, YFV, WNV and JEV. At the same time, the result for the RT-LAMP assay for field-collected mosquito samples was consistent with RT-PCR and previously reported metagenomic analysis data, the mosquito samples with abundant BAV reads in metagenomic analysis were detected positively by RT-LAMP (Atoni et al., 2018; Xia et al., 2018b). In addition, this assay is fast and convenient. Positive results can be obtained within 40 min, and the device is user-friendly, with no need for special training to conduct the assay or interpret the results. Unfortunately, the real clinical sample for BAV was absent in the current study because a confirmed BAV infection case is rarely reported. But we will use clinical samples from patiens with unknown fever or encephalitis to evaluate the established RT-LAMP assay in the future work.

Table 3
Detection of BAV in field collected mosquito samples in China.

Collection date	Region	No. of pools (30–50 mosquitos/pool) and species	No. for positive pools	
			RT-LAMP	Conventional RT-PCR
2014	Hubei	10 (*Culex quinquefasciatus*)	1	1
		5 (*Anopheles sinensis*)	1	1
2015	Yunnan	17 (*Culex tritaeniorhynchus*)	4	4
2017	Guangzhou	15 (*Aedes albopictus*)	0	0

The possible disadvantages of the RT-LAMP assay are more expensive costs for reagents, and the potential for aerosol contamination when opening the tube after the reaction because of the large amounts of material required for RT-LAMP production. In our assay, both in the real-time and visual tests, the result could be assessed with no need for opening the reaction tube. This result maintains a clean environment for the reaction preparation and allows for careful manipulation, which works well to avoid contamination.

In summary, we successfully developed a RT-LAMP assay for the detection of BAV, which provides a potential new molecular diagnostic test for BAV that could be applied in the field or clinic in the future, and that may contribute to the preparedness for future outbreaks of a BAV endemic, especially for regions with limited resources available.

Conflicts of interest

No conflict of interest.

Funding source

This work was supported by the Ministry of Science and Technology of the People's Republic of China [2018ZX10101004, 2013FY113500]; the Science and Technology Bureau of Wuhan, China [2018-201261638501]; the Chinese Academy of Sciences [153211KYSB20160001, ZDRW-ZS-2016-4] and the Wuhan Institute of Virology, China [WIV-135-PY2].

Ethical approval

This article does not contain any studies with human or animal subjects performed by any of the authors.

Acknowledgment

We are grateful to the Hubei Provincial Center for Disease Control and Prevention (Hubei CDC) for assistance in mosquito sampling.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijid.2018.10.025.

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