Arch Virol (2012) 157:1779–1783
DOI 10.1007/s00705-012-1348-1

BRIEF REPORT

Development and evaluation of a reverse transcription loop-mediated isothermal amplification assay for rapid detection of a new SFTS bunyavirus

Guoliang Yang · Bing Li · Lanzheng Liu · Weichu Huang · Wen Zhang · Yuandong Liu

Received: 30 November 2011 / Accepted: 19 April 2012 / Published online: 30 May 2012

Abstract The etiological agent of severe fever with thrombocytopenia syndrome (SFTS) is a bunyavirus that was first identified in China in 2009. We have developed and validated a one-step, single-tube, reverse-transcription loop-mediated isothermal amplification (RT-LAMP) assay for detection of SFTS bunyavirus (SFTSV). This assay demonstrated high specificity and sensitivity, with a detection limit of 10^1 $TCID_{50}$ ml^{-1}. When combined with the fluorescent detection reagent (FDR) method, results could be determined by observing a color change within 30 min. As an accurate, rapid, simple and low-cost diagnostic method, this RT-LAMP assay will be helpful for detecting and preventing further SFTSV infection in China.

Keywords Clinical diagnosis · Severe fever with thrombocytopenia syndrome bunyavirus (SFTSV) · Reverse transcription loop-mediated isothermal amplification (RT-LAMP) · Family *Bunyaviridae*

A hemorrhagic fever-like illness was observed in March 2009 in central China. The fatality rate of this disease was very high (about 12-30 %) [1–4]. The main clinical manifestations of this disease included high fever, gastrointestinal symptoms, thrombocytopenia, and low counts for white blood cells. A new kind of bunyavirus, designated severe fever with thrombocytopenia syndrome bunyavirus (SFTSV), was identified to be the pathogen of this illness [1]. This novel tick-borne virus belongs to the genus *Phlebovirus* in the family *Bunyaviridae*.

Based on data from an epidemiological investigation, of all SFTSV infection cases, over 97 % were farmers working and living in remote villages. The medical resources in these regions are limited. To save lives, it is important to develop a rapid, accurate, and low-cost method for SFTSV detection. The current SFTSV detection methods involve the detection of IgM and IgG by enzyme-linked immunosorbent assays (ELISAs), an SFTSV neutralization test, virus isolation, and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) with high specificity and sensitivity [4]. However, these methods might not fully meet the requirements of SFTSV diagnosis because they are labor-intensive and time-consuming, and there is a need for expensive regents, well-trained technicians, and precision equipment. Recently, a novel nucleic acid amplification method designated loop-mediated isothermal amplification (LAMP) has been developed [5]. It is based on a strand-displacement reaction, which occurs under isothermal conditions. Because it uses six oligonucleotide primers to recognize eight regions on the target DNA, amplification by the LAMP method is highly specific [5–7]. This method can amplify a few copies of target DNA to a significant amount in 60 min with no expensive reagents required. Combined with reverse transcription, the reverse transcription-LAMP

G. Yang and B. Li contributed equally to this paper.

Electronic supplementary material The online version of this article (doi:10.1007/s00705-012-1348-1) contains supplementary material, which is available to authorized users.

G. Yang · L. Liu · W. Zhang
Jinan Center for Disease Control and Prevention, Jinan 250021, Shandong Province, China

B. Li (✉) · W. Huang · Y. Liu
Jinan Junqu Center for Disease Control and Prevention, No. 36 Wen Hua Dong Lu Street, Jinan 250014, Shandong Province, China
e-mail: ecolilee@yahoo.com.cn

(RT-LAMP) method is able to detect target RNA in one step [8]. As a cheap, rapid and simple diagnostic method, the LAMP assay is suitable for application in developing countries. To date, LAMP methods for the detection of many pathogens, such as viruses, bacteria and parasites, have been developed. These include the 2009 pandemic influenza A H1N1 virus [9], enterovirus 71 [10], West Nile virus [11], and Ebola virus [12]. All of these methods showed sufficient to high specificity, efficiency and sensitivity in comparison with PCR assays and many other conventional detection methods.

In this study, a simple RT-LAMP assay targeting SFTSV was developed and evaluated. The diagnostic results of this RT-LAMP assay could be determined directly by the naked eye based on color difference. The clinical diagnostic ability of this method was assessed. Our study results showed that the SFTSV-specific RT-LAMP assay is sensitive and accurate and has the potential to be a useful complement in SFTSV diagnostics.

Six SFTSV strains (strains JN001, JN002, JN003, JN004, JN005 and JN006; 10^4 50 % tissue culture infective dose [$TCID_{50}$] per ml) isolated from confirmed SFTSV infection cases were used in this study. These strains were isolated by our laboratory during SFTSV outbreaks that occurred in central China. Their identities were confirmed by qRT-PCR [13] and virus isolation methods. Vero cells were grown at 37 °C/5 % CO_2 in minimal essential medium (MEM; Gibco, NY, USA) supplemented with 10 % fetal calf serum. The SFTSV-infected Vero cell cultures were titered, and their $TCID_{50}$ values were determined according to previously described methods [1, 13]. To evaluate the specificity of this assay, other bunyaviruses and pathogens that cause similar symptoms were used in our experiments (Table 1). Viral RNA specimens were extracted from virus suspensions (200 µl) using a Roche High Pure Viral RNA Kit (Roche, Mannheim, Germany) in accordance with the manufacturer's instructions. The RNA was dissolved in 50 µl DEPC-treated water and stored at −80 °C. All SFTSV culture and RNA extraction procedures were done in Biosafety Level 2 facilities.

To design the SFTSV-specific primers, all available SFTSV L/M/S segment sequences in GenBank were retrieved and analyzed using the ClustalX alignment programs [14] to identify conserved regions (accession numbers of these SFTSV segments are given in Online Resource 1). Three sets of six SFTSV-specific primers were designed using LAMP primer design software (PrimerExplorer V4; http://primerexplorer.jp/elamp4.0.0/index.html) as described previously by Notomi et al. [5]. The practicality and specificity of all primer sets were evaluated by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and tested with different SFTSV isolates. After these preliminary screens, the best-performing primer set was selected. This primer set consisted of two outer (F3_S, 5′-CAG ATA CCC CCG CAG TTG-3′ and B3_S, 5′-AGA GGT TGA TGG CAC TCC A-3′), two inner (FIP_S, 5′-TAT CTC CCA GTG GGG CCA GCT CCA AAG GCC ATG CAC ATC-3′ and BIP_S, 5′-GTG GGA AGG CTC TGC GCT ACT GAG AGG GCA GAA ACC AGG-3′) and two loop (LF_S, 5′-AAG GTT GAG AAT TAT CCC CCT G-3′ and LB_S, 5′-CCT CAC AGG AGT GAT TGA GAG-3′) primers that recognize eight distinct regions on the S segment of SFTSV strain LN3. (S segment: HQ141612.1). The details of this primer set are shown in Online Resource 2.

RT-LAMP was conducted using a Loopamp RNA Amplification Kit (Eiken Chemical Co., Ltd. Tokyo, Japan). The reaction was carried out in a reaction mixture (25 µl) containing 40 pmol of each of the inner primers (FIP and BIP), 5 pmol each of the outer primers (F3 and B3), 20 pmol each of the loop primers (LF and LB), 12.5 µl of 2× reaction mix, 1.0 µl of enzyme mix, and 5 µl of template RNA. Total RNA extracted from uninfected

Table 1 Pathogens used in specificity evaluation

Pathogen	Strain	Concentration
SFTSV	Strains JN001 to JN006	10^4 $TCID_{50}$ ml^{-1}
HFRS Hantaan virus	Strain Z10	$10^{6.0}$ $CCID^{50}$ ml^{-1}
HFRS Hantaan virus	Strain Z37	$10^{6.0}$ $CCID^{50}$ ml^{-1}
Yellow fever virus	Strain 17D	>3.66 log LD_{50}
Japanese encephalitis virus	Strain SA14-14-2	>5.7 log PFU ml^{-1}
Human enterovirus 71	HEV71/Jinan035/SD/CHN/2011(C4)	$10^{6.5}$ $TCID_{50}$ ml^{-1}
Coxsackievirus A16	COXA16/Jinan034/SD/CHN/2011(B1)	$10^{6.1}$ $TCID_{50}$ ml^{-1}
Influenza virus	A/Shandongtianqiao/SWL192/2011(H1N1)	$10^{6.0}$ $TCID_{50}ml^{-1}$
Influenza virus	B/Shandongzhangqiu/1171/2011(Victoria)	$10^{5.2}$ $TCID_{50}$ ml^{-1}
Rotavirus	Strain LLR	$10^{5.5}$ $CCID_{50}$ ml^{-1}
Leptospira interrogans	Strain CMCC(B) 56110	$10^{9.3}$ ml^{-1}
Human granulocytic anaplasmosis	RNA extracted from HGA-positive white blood cell specimens (SD10039, human white blood cell, China, 2010)	

Vero cell culture was used as a negative control. The positive control RNA (PC RNA) provided in the Loopamp RNA Amplification Kit was used as a positive control. The reaction mixture was incubated at 63 °C for 30 min. After the reaction, the products were analyzed by 1 % agarose gel electrophoresis. The reaction results were also confirmed by the SYBR-RT-LAMP or FDR-RT-LAMP methods.

Using the SYBR-RT-LAMP method, the LAMP reaction can be monitored by real-time follow-up, similar to qRT-PCR assays. The SYBR-RT-LAMP reaction was carried out in a Stratagene MX3005P (Agilent, Germany) by adding 1 μl of a 10^{-4} dilution of SYBR Green I solution (TIANGEN, Beijing, China) with fluorescence read on the FAM channel. The parameters were as follows: The sample was maintained at 63 °C for 40 min, fluorescence was read every minute, and characteristic hat-shaped amplification curves were acquired and analyzed [15]. An assay result was considered positive when the fluorescence increased above the baseline value within the 40-min incubation time.

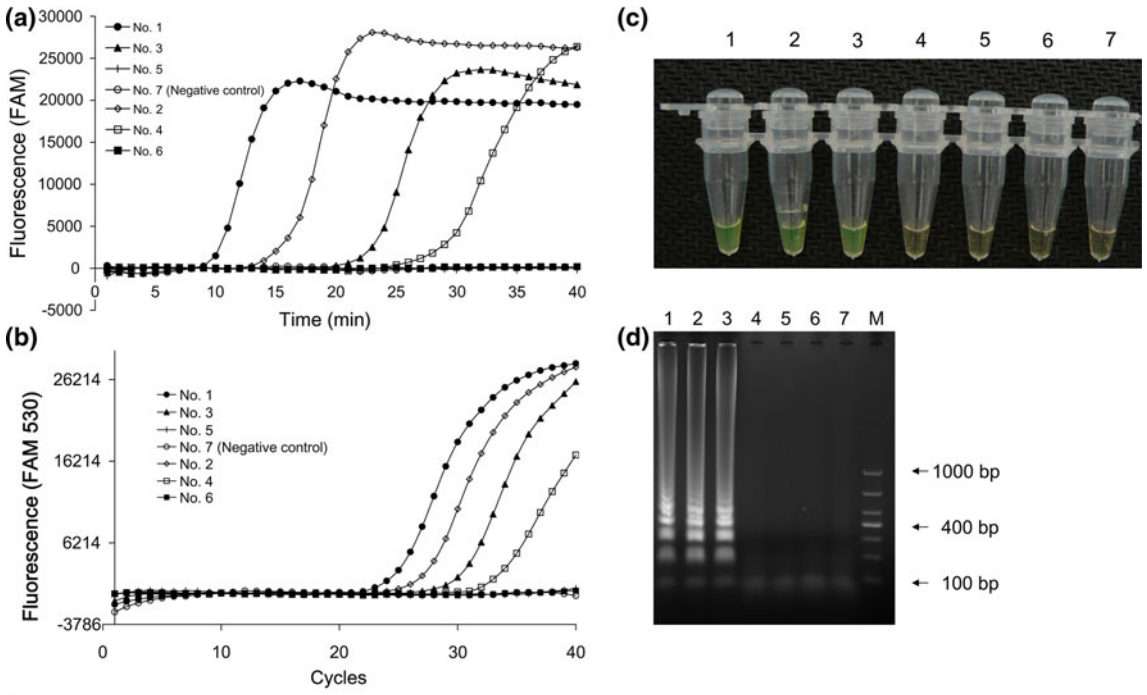
For the FDR-RT-LAMP method, the LAMP assay results could be determined directly by visual observation based on color change. Fluorescent detection reagent (1 μl; FDR; Eiken Chemical Co., Ltd., Tokyo, Japan) was added to the LAMP amplification mixture. The reaction mixtures were incubated at 63 °C for 30 min. For a positive reaction, the color of the reaction mixture changed from dark yellow to bright green within 30 min; a negative reaction was indicated when the color remained dark yellow. This color change can be observed by the naked eye under natural light or with the aid of UV light.

To assess the sensitivity of the LAMP assay, serial tenfold dilutions of RNA (10^4 to 10^{-1} $TCID_{50}$ ml^{-1}) from SFTSV strain JN001 were subjected to LAMP and qRT-PCR assays and compared. Total RNA extracted from uninfected Vero cell culture was used as a negative control. The reaction products were analyzed by 1 % agarose gel electrophoresis (Gel-RT-LAMP). Additionally, to compare the sensitivity of the three different LAMP analysis methods, the same templates were also detected by SYBR-RT-LAMP and FDR-RT-LAMP. The qRT-PCR assay for SFTSV was conducted according to Sun et al. [13]. All tests were repeated two times. The results showed that the SYBR-RT-LAMP assay demonstrated a detection limit of approximately 10^1 $TCID_{50}$ ml^{-1} (Fig. 1a), equal to that for the qRT-PCR (Fig. 1b). When determining the LAMP results by the FDR visual detection method, a detection limit of around 10^2 $TCID_{50}$ ml^{-1} of virus RNA was determined (Fig. 1c). The Gel-RT-LAMP assay had the same detection limit as the FDR method (Fig. 1d). According to these results, the sensitivity of the SYBR-RT-LAMP method was similar to that of the qRT-PCR assay. The sensitivity of the FDR-LAMP assay was equal to that of the Gel-LAMP, and ten times lower than for the real-time SYBR reaction and qRT-PCR methods (Fig. 1e).

The specificity of the LAMP assay was evaluated by cross-reactivity tests with other bunyaviruses and related viruses, which possibly cause similar symptoms. The RT-LAMP assay was also tested against RNA extracted from other pathogens as outlined above. The RT-LAMP results were determined by the FDR visual detection method and agarose gel electrophoresis. No positive results were observed using these methods except for six SFTSV strains (Online Resource 3).

To evaluate the clinical diagnostic capability of the FDR-RT-LAMP assay, detection was carried out with clinical sera (n = 53) and healthy human sera (n = 30) in a blinded manner. Of the 53 clinical cases, 20 were SFTSV-positive patients, 17 were non-SFTSV patients who exhibited similar symptoms, and 16 were patients that had been in close contact with SFTS patients. The patients from whom clinical samples were obtained remained anonymous. Local ethical approval was obtained, and international guidelines were followed for the use of clinical materials and access to diagnostic results. Total RNA from each serum sample was extracted manually using a Roche High Pure Viral RNA Kit. The RNA solution (5 μl) was added to the LAMP reaction tubes as the template. The color of the reaction mixture changed from yellow to bright green within 30 min, and this was considered a positive result. Every detection result was validated using the SYBR-RT-LAMP method. All serum samples were previously confirmed by virus isolation and qRT-PCR according to the 'Guidelines for the diagnostic and treatment of SFTSV 2010', issued by the Chinese Center for Disease Control and Prevention.

The clinical evaluation results are presented in Table 2. Eighteen positive serum samples were confirmed by the FDR-RT-LAMP assay, while 20 serum samples were positive by the qRT-PCR and SYBR-RT-LAMP assays. Two positive serum samples were not detected by the FDR method. According to these results, the sensitivity of the FDR-RT-LAMP assay in this experiment was 90 %, the specificity was 100 %, and the diagnostic accuracy rate was 97.59 %. Among all 63 non-SFTSV samples, zero false positive amplification results were observed, indicating the high specificity of the RT-LAMP method. The negative predictive value (NPV) of this FDR-RT-LAMP assay was 96.92 %. The positive predictive value (PPV) of this assay was 100 %. In the experiment, two SFTSV-positive serum samples were not detected by the FDR method (PJN2011001 and PJN2011023). Based on the specimen records, most of the positive sera were collected on days 1 to 11 after the onset of symptoms, but the sera



(e) The sensitivity testing results

Sample	SFTSV Titer[a]	FDR-assay[b]	Gel-assay[b]	SYBR-assay[b]	qRT-PCR assay[b]
1	10^4	+	+	+	+
2	10^3	+	+	+	+
3	10^2	+	+	+	+
4	10	-	-	+	+
5	1	-	-	-	-
6	0.1	-	-	-	-
7	Neg[c]	-	-	-	-

[a] $TCID_{50}$ ml^{-1} of SFTSV in template solution
[b] +, positive test result; -, negative test result
[c] Negative control

Fig. 1 Detection sensitivity of the (**a**) SYBR-RT-LAMP assay, (**b**) qRT-PCR assay, (**c**) FDR-RT-LAMP assay, and (**d**) Gel-RT-LAMP assay. These were tested using serial tenfold dilutions of SFTSV RNA extracted from supernatants of infected cells. The titers of these six specimens ranged from 10^4 to 10^{-1} $TCID_{50}$ ml^{-1}. Specimen 7 represents the negative control. The reaction tubes of the FDR-RT-LAMP assay and Gel-RT-LAMP assay were incubated at 63 °C for 30 min. (**d**) *M*, DNA marker DL-1000. (**e**) Summary table of all sensitivity testing results

PJN2011001 and PJN2011023 were collected on days 13 and 15 after onset. The SFTSV titer in these serum samples was calculated from the C_t value of the qRT-PCR assay. The SFTSV titer was 23.99 and 8.31 $TCID_{50}$ ml^{-1} for PJN2011001 and PJN2011023, respectively. These titers were lower than the detection limit of the FDR-RT-LAMP method but still within the detection range of the qRT-PCR and SYBR-RT-LAMP assays. This may be the reason why these two samples could not be detected by the FDR assay, yet positive results were observed by the SYBR method and qRT-PCR assay. These results indicate that the optimal sample collection time for application in the FDR-RT-LAMP assay is best restricted to within 10 days after the onset of symptoms. This result is in accord with previous studies regarding SFTSV-specific qRT-PCR assays [13].

In this study, we have developed a novel RT-LAMP assay as a diagnostic method for detecting SFTSV. There

Table 2 Evaluation of clinical detection by FDR-RT-LAMP and SYBR-RT-LAMP assays

Serum group	Results	FDR-RT-LAMP	SYBR-RT-LAMP	qRT-PCR	Virus isolation
SFTS patients	Pos[a]	18	20	20	7
	Neg[b]	19	17	17	30
	Total	37	37	37	37
Close contacts	Pos	0	0	0	0
	Neg	16	16	16	16
	Total	16	16	16	16
Healthy donors	Pos	0	0	0	0
	Neg	30	30	30	30
	Total	30	30	30	30

[a] Pos, positive detection of SFTSV
[b] Neg, SFTSV was not detected

has been a recent and significant increase in epidemics of SFTSV in China. SFTSV infections have been reported in seven provinces in central, northeast and eastern China. Rapid detection of SFTSV is extremely important for timely diagnosis, disease prevention and disease surveillance. Additionally, as it is a tick-borne virus, most SFTSV infections occur in remote villages and farms. The medical resources in these regions are limited, and complicated or expensive diagnostic methods cannot be effectively carried out. To save lives, a detection method such as RT-LAMP that is simple, cheap, rapid and sensitive would be ideal for practical applications in rural areas and in the field.

In developing a rapid detection method, specificity and sensitivity are also critical. In this research, the SYBR-RT-LAMP method demonstrated the highest sensitivity, as it could detect 10 $TCID_{50}$ ml^{-1} of SFTSV. This was of equal sensitivity to the qRT-PCR assay and 10 times higher than that observed for the FDR-RT-LAMP and gel assays. However, expensive and bulky instruments are needed to carry out the SYBR-RT-LAMP assay. In contrast, the FDR-RT-LAMP assay could be carried out on a simple heating block, and the reaction results could be determined directly by the naked eye. The FDR-RT-LAMP method is therefore more suitable for application under conditions where resources are limited. Furthermore, the FDR dye was added during the preparation of reaction mixture, and the reaction results could be determined directly based on color change without opening the lids of the reaction tubes. This aspect of the assay could prevent contamination, which is extremely important for sequence-dependent detection methods. Therefore, when conducting the FDR-RT-LAMP assay, the LAMP detection process is significantly simplified and can be easily facilitated under field conditions.

When assessing the specificity of this assay, high specificity was demonstrated by the FDR-RT-LAMP assay. The primers were able to amplify all SFTSV strains but not the 11 other related pathogens. This result is consistent with other reports regarding the specificities of LAMP assays [9–11].

In conclusion, the RT-LAMP assay for detection of SFTSV is cheap, rapid, and accurate. In particular, the FDR-RT-LAMP assay demonstrated high specificity and sensitivity without the requirement for very specific and expensive equipment and complicated techniques. The results of the FDR assay could be determined directly based on color change without opening the lids of reaction tubes. The FDR-RT-LAMP assay employs regular primers without the requirement for labeled probes, so the cost for each LAMP reaction is seven times lower than for a standard qPCR assay [16]. The many advantages of this FDR-RT-LAMP assay make it suitable and useful for field detection of SFTSV during the early stages of infection, assisting in the control of and prevention of SFTSV infection.

References

1. Yu XJ, Liang MF, Zhang SY, Liu Y, Li JD, Sun YL, Zhang L, Popov VL, Li DX et al (2011) Fever with thrombocytopenia associated with a novel bunyavirus in China. N Engl J Med 364:1523–1532
2. Zhang YZ, Zhou DJ, Xiong Y, Chen XP, He YW, Sun Q, Wang Y, de Jong MD, Xu J et al (2011) Hemorrhagic fever caused by a novel tick-borne Bunyavirus in Huaiyangshan China. Zhonghua Liu Xing Bing Xue Za Zhi 32:209–220
3. Stone R (2010) Rival teams identify a virus behind deaths in central China. Science 330:20–21
4. Li DX (2011) Fever with thrombocytopenia associated with a novel bunyavirus in China. Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi 25:81–84
5. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28:1–7
6. Tomita N, Mori Y, Kanda H, Notomi T (2008) Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat Protoc 3:877–882
7. Nagamine K, Hase T, Notomi T (2002) Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol Cell Probes 16:223–229
8. Fukuta S, Iida T, Mizukami Y, Ishida A, Ueda J, Kanbe M, Ishimoto Y (2003) Detection of Japanese yam mosaic virus by RT-LAMP. Arch Virol 148:1713–1720
9. Ma XJ, Shu YL, Nie K, Qin M, Wang DY, Gao RB, Wang M, Wen LY, Han F, Zhou SM, Zhao X, Cheng YH, Li DX, Dong XP (2010) Visual detection of pandemic influenza A H1N1 Virus 2009 by reverse-transcription loop-mediated isothermal amplification with hydroxynaphthol blue dye. J Virol Methods 167: 214–217
10. Tao J, Juan L, Yongqiang D, Lijuan X, Xiaofeng L, Han J, Cao RY, Qin ED, Qin CF (2011) Development and evaluation of a reverse transcription-loop-mediated isothermal amplification assay for rapid detection of enterovirus 71. J Clin Microbiol 49:870–874
11. Parida M, Posadas G, Inoue S, Hasebe F, Morita K (2004) Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of West Nile virus. J Clin Microbiol 42:257–263
12. Kurosaki Y, Takada A, Ebihara H, Grolla A, Kamo N, Feldmann H, Kawaoka Y, Yasuda J (2007) Rapid and simple detection of Ebola virus by reverse transcription-loop-mediated isothermal amplification. J Virol Methods 141:78–83
13. Sun Y, Liang M, Qu J, Jin C, Zhang Q, Li J, Jiang X, Wang Q, Lu J, Gu W, Zhang S, Li C, Wang X, Zhan F, Yao W, Bi Z, Wang S, Li D (2011) Early diagnosis of novel SFTS bunyavirus infection by quantitative real-time RT-PCR assay. J Clin Virol. doi: 10.1016/j.jcv.2011.09.031
14. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948
15. Peyrefitte CN, Boubis L, Coudrier D, Bouloy M, Grandadam M, Tolou HJ, Plumet S (2008) Real-time reverse-transcription loop-mediated isothermal amplification for rapid detection of rift valley fever virus. J Clin Microbiol 46:3653–3659
16. Pyrc K, Milewska A, Potempa J (2011) Development of loop-mediated isothermal amplification assay for detection of human coronavirus-NL63. J Virol Methods 175:133–136