VIROLOGICA SINICA, October 2012, 27 (5):273-277
DOI 10.1007/s12250-012-3252-y

Research Article

Rapid Detection of Filoviruses by Real-time TaqMan Polymerase Chain Reaction Assays*

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Ebola virus (EBOV) and Marburg virus (MARV) are causative agents of severe hemorrhagic fever with high mortality rates in humans and non-human primates and there is currently no licensed vaccine or therapeutics. To date, there is no specific laboratory diagnostic test in China, while there is a national need to provide differential diagnosis during outbreaks and for instituting acceptable quarantine procedures. In this study, the TaqMan RT-PCR assays targeting the nucleoprotein genes of the Zaire Ebolavirus (ZEBOV) and MARV were developed and their sensitivities and specificities were investigated. Our results indicated that the assays were able to make reliable diagnosis over a wide range of virus copies from 10^3 to 10^9, corresponding to the threshold of a standard RNA transcript. The results showed that there were about 10^{10} RNA copies per milliliter of virus culture supernatant, equivalent to 10,000 RNA molecules per infectious virion, suggesting the presence of many non-infectious particles. These data indicated that the TaqMan RT-PCR assays developed in this study will be suitable for future surveillance and specific diagnosis of ZEBOV and MARV in China.

Ebola virus; Marburg virus; Nucleoprotein (NP) gene; TaqMan RT-PCR

Ebola virus (EBOV) and Marburg virus (MARV) are enveloped, single-stranded, negative-sense RNA viruses classified into two genera, Ebolavirus and Marburgvirus, in the family *Filoviridae*, order *Mononegavirales*[6]. There is a single Marburgvirus species, Lake Victoria marburgvirus (LVMARV), whereas there are five known Ebolavirus species, Zaire ebolavirus (ZEBOV), Sudan ebolavirus (SEBOV), Reston ebolavirus (REBOV), Cote d'Ivoire ebolavirus (CIEBOV) and Bundibugyo (BEBOV) with ZEBOV regarded as the most virulent in humans[23]. The genomic structures of filoviruses are very similar and the genome is approximately 19 kb in length, containing seven genes arranged sequentially in the order nucleoprotein (NP)-viral protein (VP) 35-VP40-glycoprotein-VP30-VP24-RNA polymerase (L)[19].

These viruses are causative agents of severe hemorrhagic fever with high mortality rates in humans and non-human primates with unknown treatments[8]. Since the discovery of Marburg hemorrhagic fever in Germany in 1967, sporadic outbreaks of Marburg and Ebola hemorrhagic fever have been reported from different countries in Central Africa[8]. Incidences have increased in Central Africa since the beginning of the new millennium[3, 4]. Furthermore, two imported cases of Marburg hemorrhagic fever (in the Netherlands and the United States)[2,20] and one of Ebola hemorrhagic fever (in South Africa)[25] in travelers, have been reported, emphasizing the risk of filovirus infection in non-endemic countries.

Due to its potential for adverse public health impact with mass casualties, EBOV and MARV have been classified as Category-A critical biological agents[17]. These disease-causing hemorrhagic fever RNA viruses have the potential to be used as bioterrorism agents, highlighting the need for their accurate and timely identification. Previous diagnostic methods for EBOV include antigen-capture ELISA testing of serum and blood samples, followed by nested reverse-transcription PCR (RT-PCR) amplification of RNA from the captured virions; however, these methods have limited throughput capacity and need up to a day to acquire the results [7,22]. Several groups have developed real-time RT-PCR assays that have high sensitivity as well as high throughput capacity[5,11,22,24]. As rapid real-time PCR assays have become routine in diagnostic laboratories, there has been an ever-increasing number of commercially

Received: 2012-04-10, Accepted: 2012-08-06
* Foundation items: Supported by Important National Science &Technology Specific Projects (2009ZX10004-504, 2009ZX09301-014); National Natural Science Foundation of China (81072675).
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available reagent systems and detection platforms to support these methods.

In China, increasing personal exchange with other counties where sporadic outbreaks have been recorded raises the concerns of potential outbreaks of exotic viruses, however, no reliable diagnostic method has been established for the surveillance of such viruses. In this study, the TaqMan-based, real-time reverse transcription–polymerase chain reaction (RT-PCR) assays targeting the nucleoprotein (NP) genes of ZEBOV and MARV were developed and the sensitivities and specificities were studied. The results indicate that the assays can rapidly detect and identify the gene targets of the genomes of these two filoviruses, which suggests the potential use as a standard detection method in the diagnostics and epidemiological studies of ZEBOV and MARV infections, and may play a role in the development of vaccines and antiviral drugs of these viruses.

MATERIALS AND METHODS

Biosafety procedures

Laboratory procedures for the propagation of EBOV and MARV were performed in the Laboratoire P4 Jean Mérieux in Lyon with requisite containment and safety precautions.

Cells and viruses

Virus stock was prepared in the BSL-4 laboratory by infecting Vero cells with a multiplicity of infection (MOI) of 0.01 plaque forming units (pfu)/cell and virus was recovered 5 days post-infection. Supernatants from mock and virus infected cells were transferred into Eppendorf tubes, clarified by centrifugation at 2000 rpm for 5 min and split into fresh tubes. One was treated for RNA extraction and quantification and a second one used for virus titration.

Virus titration

Viruses were titrated by plaque assay on Vero cells. Briefly, six-well plates containing Vero cells were incubated for 1 h at 37 ºC in a 5 % CO_2 incubator with 1 mL of serial 1:10 dilutions of virus. Cells were washed twice with Dulbeco's minimum essential medium (DMEM) without fetal calf serum (FCS) and covered with 2 mL of 1.6 % carboxymethylcellulose in DMEM containing 5 % FCS. After incubation for 4–5 days at 37 ºC in 5 % CO_2, the medium was removed, and the cells were washed with PBS and fixed with 10 % buffered-PBS formalin. To detect viral antigen, cells were incubated with virus-specific rabbit sera, followed by addition of HRP-conjugated anti-rabbit goat IgG antibody (SIGMA). Plaques were counted and the titer expressed as pfu/mL after DAB (3,3'-iaminobenzidine, tetrahydrochloride) incubation (SIGMA).

RNA extraction

Viral RNA was extracted from 140 μL of supernatant from virus-infected Vero cells using the RNA extraction kit (Qiagen Inc., USA) following the manufacturer's instructions. The extracts were resuspended in 60 μL of AVE Buffer, aliquoted and stored at -70 ºC before RT-PCR amplification was carried out.

Preparation of *in vitro* RNA transcripts

The ZEBOV and MARV *in vitro* RNA transcripts were synthesized using the T7 RiboMAXTM expression large-scale RNA production system (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, following RT-PCR amplification of the partial NP gene (primers are listed in Table 1), the products underwent phenol: chloroform extraction and ethanol precipitation[18], and the resulting DNA pellets were resuspended in 30 μL of Tris-EDTA buffer. The *in vitro* transcription reactions were carried out for 30 min at 37 ºC with 2 μg DNA. Two units of RQ1 RNase-free DNase (Promega) were then added and incubation was continued for a further 60 min. The preparations were incubated for 15 min at 70 ºC to inactivate the DNase. The transcripts were extracted using an RNeasy mini kit (Qiagen, Hilden, Germany), and resuspended in 50 μL of DEPC-treated water. The concentration of RNA transcript was determined by measuring the optical density (OD) at 260 nm. RNA transcripts were stored at -70 ºC for further use.

Real-time RT-PCR analysis

The primers and probes, which were used in these 2 assays for targeting the NP genes of ZEBOV and MARV, are listed in Table 1. The conserved fragments of the NP genes were chosen after alignment using ClustalW[15] and the primers and probes were designed using the Primer Premier software package version 5.0 (Fig. 1).

Quantitative RT-PCR assays were performed using the DNA

Table 1. Primers and probes used in the study.

Virus	Target	Primer/Probe	Amplicon	Sequence (5'-3')
ZEBOV	NP	enpT-F	485bp	TAATACGACTCACTATAGGGATGCCGGAAGAGGAGACAA
		enpT-R		TAATACGACTCACTATAGGGCGGGCGAAAGGAGCATA
		enp-F	80bp	GCAGAGCAAGGACTGATTCA
		enp-R		GTTCGCATCAAACGGAAAAT
		enp-Probe		FAM-CAACAGCTTGGCAATCAGTTGGACA-TAMRA
MARV	NP	mnpT-F	463bp	TAATACGACTCACTATAGGGACAGGCCACATGAAAGTAAT
		mnpT-R		TAATACGACTCACTATAGGGGTGCCGCCTCTCGTAG
		mnp-F	80bp	GGTCAAACTAGATTCTCAGGACTTC
		mnp-R		GTCACCCCTGAATCAGTTTTTT
		mnp-Probe		FAM-TGTGAAAACAGTTCTCGAGTTCATC-TAMRA

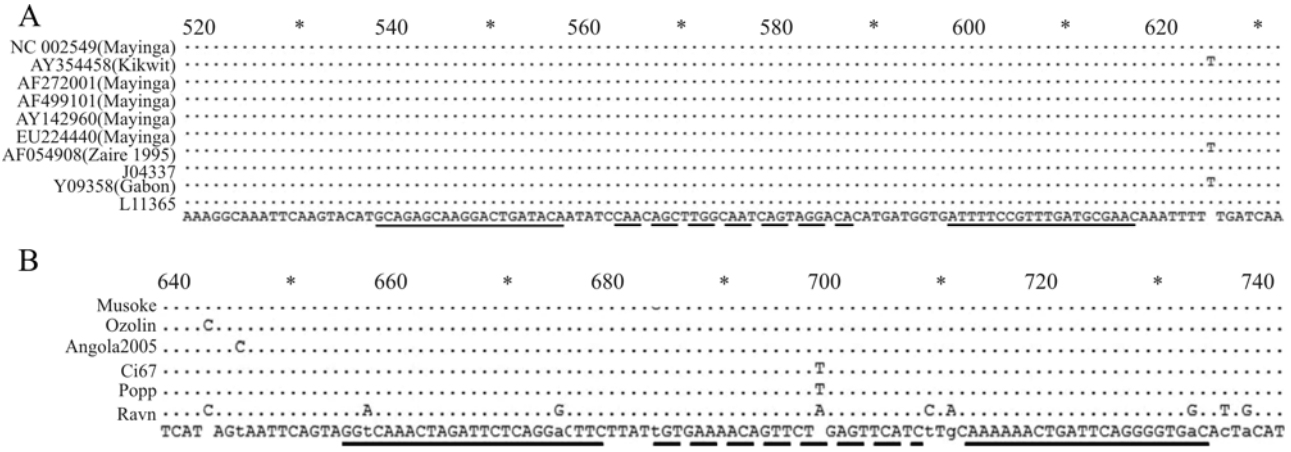

Fig. 1. Alignment of ZEBOV and MARV NP gene sequences and TaqMan RT-PCR primer and probe sequences used in this study. Dots indicate the positions identical to ZEBOV strain, Mayinga (NC 002549) or MARV strain Musoke (NC_001608) sequences. The numbers indicate the respective nucleotide positions in the Mayinga and Musoke strain genome sequences. Genbank accession numbers of the nucleotide sequences used in this study are NC 002549 (Mayinga), AY354458 (Kikwit), AF272001, AF499101, AY142960, EU224440, AF054908, J04337, Y09358, L11365 and NC 001608 (Musoke), AY358025 (Ozolin), DQ447656 (Angola), EF446131 (Ravn), EF446132 (Ci67), Z29337 (Popp).

Engine Opticon 1 system and a Roche LightCycle 2.0 Instrument. The one-step RT-PCR system (QIAGEN one-step RT-PCR Kit, Qiagen, Hilden, Germany) was used for uninterrupted thermal cycling. A master mix reaction was prepared and dispensed in 22-μL aliquots into accompanying thin-walled microAmp optical tubes. Then 1-3 μL of RNA transcript or RNA extract from stock virus or infected cell supernatants was added to each tube. The final reaction mixture contained 400 nmol/L of each primer and 100 nmol/L of the probe. Prior to amplification the RNA was reverse transcribed at 50 ºC for 30 min. This was followed by one cycle of denaturation at 94 ºC for 5 min. Next, PCR amplification was carried out for 45 cycles at 94 ºC for 15 s and 60 ºC for 1 min. The fluorescence was read at the end of this second step allowing a continuous monitoring of the amount of RNA. Amplification products were later confirmed by sequencing.

RESULTS

Sensitivity and specificity of the assays

The sensitivity of the ZEBOV and MARV TaqMan RT-PCR detection assays was evaluated by using the serial dilutions of known amounts of RNA transcribed *in vitro* from PCR products. A threshold cycle (Ct) value was calculated from the amplification plot covering this range of dilutions (Fig. 2) and two assays using transcript RNAs were used to draw a standard curve (Fig. 3). The linearity of the curve allowed quantification of viral RNA molecules in a wide range from 10^4 to 10^{10} per reaction (ZEBOV) or 10^3 to 10^9 per reaction (MARV). Moreover, the intra-assay coefficient of variation calculated by comparing the Ct values obtained for two RNA transcripts was found to vary between 0.03 % and 1.7 % and the low deviation indicates that the assay is highly reproducible. Similar data were obtained when the test was repeated three times, underlining the reproducibility of the assays (data not shown).

The specificity of the assays was verified by the existence or absence of amplification using MARV viral RNA corresponding to ZEBOV primers and probe or vice versa (Fig. 4).

Quantitation of virus load in infected cell supernatants

To determine the accuracy of our TaqMan RT-PCR method for quantitation of MARV RNA, infectious virus titers were calculated by TaqMan RT-PCR using a RNA transcript standard curve (Fig. 4). Vero cells were infected with MARV at a multiplicity of infection of 0.01 pfu/cell. Complete cell destruction was observed 5 days post-infection and cell supernatants taken at that time were analyzed (Fig. 5). The quantitation results showed that there were about 10^{10} viral RNA copies per mL of MARV infected cell supernatant.

Similarly, vero cells were infected with ZEBOV at MOI of 0.01 pfu/cell. Cell supernatants taken at 5 days post-infection were used for RNA extraction and quantification (Fig. 4) as well as virus titration (~10^6 pfu/mL, Fig. 5). From the RNA transcript standard curve and the TaqMan RT-PCR Ct value, it could be deduced that the viral RNA copies of ZEBOV were to those of MARV, at 10^{10} copies/mL. Therefore it could be concluded that the number of viral genome molecules was about 4 logs higher than the number of live infectious virions.

DISCUSSION

TaqMan RT-PCR assays were developed in this study for rapid, accurate and quantitative diagnosis of ZEBOV and MARV infection. These tests fill a capability gap for the diagnosis and response to potential filovirus infection in China. They could detect a wide range of virus concentrations from 10^9 copies to 10^3 copies per reaction. This sensitivity is similar, if not higher, with that from other filovirus detection assays published previously[1].

The reproducibility of the TaqMan RT-PCR assays was high since only small variations were observed in the results obtained

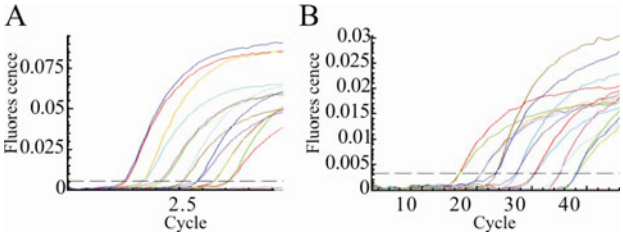

Fig. 2. Detection sensitivity of the ZEBOV (A) and MARV (B) TaqMan RT-PCR system. Amplification plots were based on 10-fold dilutions of transcript RNAs. Tests were performed in duplicate from 10^{-2} to 10^{-8}.

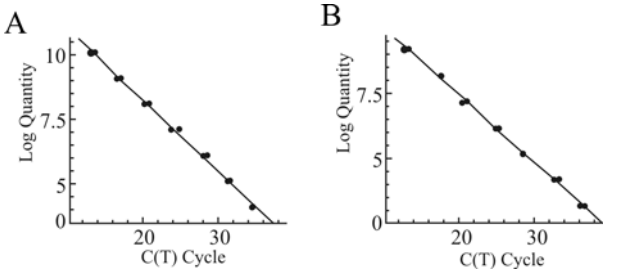

Fig. 3. Standard curves obtained with 10-fold serial dilutions of ZEBOV and MARV RNA transcribed *in vitro* from PCR products. Ct values calculated from results in Fig. 1 are plotted against the log of the initial starting quantity of RNA transcript (copies/uL). The threshold is 0.28(A) and 0.29(B), respectively.

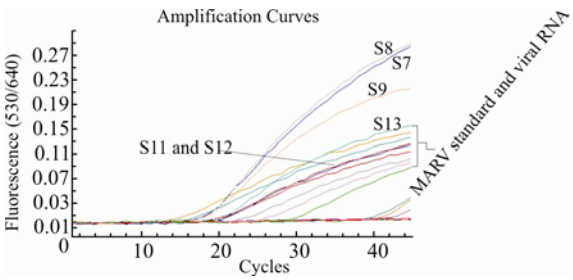

Fig. 4. Quantitation of virus load in infected cell supernatants using a Roche Light Cycle system in the Biosafety level (BSL) 4 Jean Merieux laboratory in Lyon. The MARV standard curves were obtained with 10-fold serial dilutions of MARV RNA transcribed in vitro from PCR products. Sample S11 and S12 were MARV RNA extracted from infected cell supernatants and S13 was MARV RNA transcript (as positive control). The sample S7 and S8 were ZEBOV RNA extracted from infected cell supernatants, and sample S9 was ZEBOV RNA transcript (as positive control). These assay included the existence or absence of amplification and mock-infected cell supernatant using MARV viral RNA corresponding to ZEBOV primers and probe or using ZEBOV viral RNA corresponding to MARV primers and probe all showed no amplification curve.

from several tests conducted at different times and with different RNA preparations (Fig. 2 and Fig. 3). Thus the reliability of the test will principally depend on the quality of RNA samples and the method of RNA extraction. RNA transcripts were developed as stable, reproducible and reliable

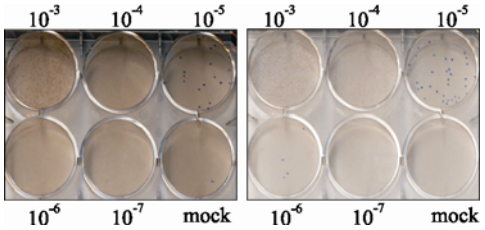

Fig. 5. ZEBOV titration. Plaques produced by ZEBOV on monolayers of Vero cells.

standards for quantitative assays. The linear range of MARV RNA quantitation was 10^3 to 10^9 copies per reaction and similar results were obtained for ZEBOV. Though the liner range was at least 10^4 to 10^{10} copies per reaction in our test, it is reasonable to assume that our ZEBOV TaqMan RT-PCR system could detect fewer RNA molecules based on the amplification curve and Ct value of the last dilution (10^4 copies). This range of linearity allows the detection of a wide range of virus titers and should permit identification and quantitation of ZEBOV and MARV in clinical specimens and in cell cultures samples without the need for titration.

The number of viral genome molecules calculated by TaqMan assay was found to be about 4 logs higher than the corresponding number of infectious virus particles measured by plaque titration. For dengue virus, it was also found that each infectious pfu correspond at least to 100 or more genomic equivalents and for Rift Valley Fever or Puumala virus a 2–3 log difference was noted[9,10,13]. This ratio is due to the presence of non-infectious virus, either defective, immature, or inactivated particles or a consequence of free viral RNA material released from damaged infected cells.

Rapid TaqMan RT-PCR diagnostics plays an important role in surveillance and case management[14,16]. During the Ebola outbreak (Zaialre ebolavirus) in 2003 in Mbomo, the Ministry of Health of the Republic of Congo together with partners from the Centre National de la Recherche Scientifique et Muséum National d'Histoire Naturelle, France and the Bernhard-Nocht Institute for Tropical Medicine, Germany, operated a small field laboratory under the lead of WHO using antigen capture and qRT-PCR to diagnose acute cases[7].

On the other hand, PCR-based assays, like other diagnostic tests, have weaknesses and do not produce reliable results under all circumstances. PCR based techniques can be prone to contamination resulting in false positive results and the consequences of false-positive and false negative results of qPCR assays could be dire to outbreak management especially during early disease stages and for survivors in the early convalescent stage [21]. Therefore, development and application of independent, methodologically different confirmatory assays such as antigen capture to support qPCR should be mandatory.

It is common nowadays for most laboratories to use PCR as their first and most rapid diagnostic methods at least for emergency detection and screening of samples. Moreover,

during some field laboratory deployment, qRT-PCR proved to be very sensitive and reliable even in challenging environments. The combined operation of a field and reference laboratory allowed for a unique evaluation of field diagnostic capacity under difficult circumstances and proved to be accurate, efficient and safe in operation, such as during the 2005 Marburg Outbreak in Angola [12].

Acknowledgements

We are grateful to Madam Marie-Claude Georges and her colleagues for their technical assistance.

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