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Development and evaluation of a fluorogenic 5′-nuclease assay to identify Marburg virus

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The ability to rapidly recognize Marburg virus infections is critical to quickly institute proper barrier nursing precautions and limit further spread of the disease. A rapid, sensitive, and specific laboratory diagnostic test is necessary to confirm outbreaks of Marburg virus and to distinguish it from other diseases that can present with similar clinical symptoms.

A one-tube reverse transcriptase-polymerase chain reaction (RT-PCR) assay for the identification of Marburg virus was developed and evaluated using the ABI PRISM™ 7700 Sequence Detection System and TaqMan® chemistry. The sensitivity and specificity of the newly designed primer/probe set (MBGGP3) was evaluated. MBGGP3 was equivalent to or 10–100-fold more sensitive than previously designed primer sets as determined by limit of detection experiments. In addition, the MBGGP3 assay was able to detect all strains of Marburg virus tested, but gave negative results with other haemorrhagic fever and genetically related viruses. The results of this study indicate that the MBGGP3 primer/probe set is both sensitive and specific. In addition, this assay is compatible with emerging rapid nucleic acid analysis platforms and therefore may prove to be a useful diagnostic tool for the control and management of future outbreaks.

KEYWORDS: Marburg virus, filoviruses, TaqMan®, RT-PCR, diagnostic, fluorogenic 5′-nuclease assay.

INTRODUCTION

Marburg virus is a single, non-segmented, negative-stranded RNA virus belonging to the family Filoviridae. It is one of two members of the Filoviridae (Ebola virus and Marburg virus) family, which is responsible for sporadic epidemics of haemorrhagic fever in human and non-human primates.

Infection with Marburg virus results in a rapid disease progression with mortality rates of approximately 30%. The main route of filovirus transmission is contact with infected body fluids, and can occur through improper needle hygiene, direct contact with infected tissue or fluid samples, and close contact with infected patients.[1–3] Consequently, those at greatest risk for contracting the disease are health-care providers and families, which in the rural African communities may be the primary care providers for sick patients. Because of the mode of transmission, high fatality rates, and the need to institute proper barrier nursing precautions and public health measures, it is essential to establish a diagnosis early in the course of disease.[4] In regions of Africa where numerous diseases can present with similar clinical syndromes (Lassa fever, *Shigella* dysentery and malaria), the diagnosis of filoviral infections can present a challenge. During an outbreak the ability to establish a rapid diagnosis may help save the lives

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of patients presenting with treatable diseases such as *Shigella* dysentery, and malaria. In these instances, proper triage of patients depends heavily on a rapid and accurate diagnosis.

Assays currently available to diagnose Marburg virus infections include virus culture, transmission electron microscopy (TEM), immunohistochemistry, antigen-detection enzyme-linked immunosorbent assays (ELISAs), antibody-detection ELISAs, and a conventional reverse-transcriptase polymerase chain reaction (RT-PCR).[5] Each method has both advantages and disadvantages, however, to date there are few assays available that are capable of providing a sensitive, specific, and rapid diagnosis under field conditions. Virus culture is sensitive but must be performed in a biological safety level-4 (BSL-4) laboratory. TEM is a rapid technique (<3 h) but is usually only available in well-equipped research facilities.[6] Both virus culture and TEM are routinely performed by reference laboratories. Antibody-detection ELISAs are often unreliable because patients with symptomatic filoviral infections often do not mount a detectable humoral response before death.[7,8] Antigen-detection ELISAs are adequate for detecting Marburg virus infections during the acute stages of infection, however, special precautions must be taken when handling infectious samples. To date, the most rapid and potentially deployable assay that has good clinical sensitivity is based on conventional RT-PCR.

Conventional RT-PCR is a more sensitive method than antigen-detection ELISAs, and has the advantage of rendering samples non-infectious before use. However, current RT-PCR assays are not capable of detecting all known strains of Marburg virus, and they are not compatible with emerging rapid nucleic-acid analysis systems.[9] Newly designed rapid nucleic-acid analysis systems use a fluorogenic probe-based assay to monitor the accumulation of PCR product in real time.[10] Adding a probe that recognizes a complementary DNA sequence between the two primers provides an increased level of specificity to the PCR reaction. With these assays it is possible to detect a positive sample in 1 h or less. Fluorogenic probes are not incorporated into the design of conventional RT-PCR assays, therefore these assays cannot be monitored on real-time nucleic-acid analysis systems. Instead, following RT-PCR, which is routinely performed as two separate steps (an RT step and PCR step), samples must be analysed by gel electrophoresis. The total assay time for conventional RT-PCR, including data analysis by gel electrophoresis, can take 3–4 h. In this study we report the development of a fluorogenic probe-based, one-step RT-PCR assay, based on the dual RT and DNA polymerase activities of the recombinant, thermostable *Thermus thermophilus* (r*Tth*) enzyme (Applied Biosystems, Foster City, CA, USA). This assay is based on the 5′-nuclease assay which exploits the 5′ nuclease activity of r*Tth* polymerase to cleave a non-extendable, dual-labelled fluorogenic probe that is annealed to the target sequence during amplification.[11–13]

The purpose of this study was to demonstrate a highly sensitive, specific, and rapid assay that is compatible with emerging rapid nucleic-acid analysis platforms. The MBGGP3 RT-PCR assay utilizes a fluorogenic 5′-labelled probe capable of recognizing all tested strains of Marburg virus. Samples are rendered non-infectious prior to use and the risk of cross-contamination between samples is minimized because there is only one set of reagent-addition steps, and no handling of samples after PCR. The development of this assay provides a new diagnostic tool for identification of Marburg infections that may be useful for future outbreak detection and management.

MATERIALS AND METHODS

Virus growth, purification, and titres

Ebola, Marburg, and Lassa virus strains were propagated in Vero E6 cells. Crimean-Congo haemorrhagic fever (CCHF) and measles (Edmonston strain) viruses were propagated in Vero cells. Vesicular stomatitis virus (VSV) was propagated in baby hamster kidney (BHK) cells. Upon visualization of 60–70% cytopathic effects (CPE), supernatant was harvested and clarified by centrifugation. For Ebola, Marburg, and measles virus purification, the supernatant was clarified in a Sorvall centrifuge at 1500 × ***g*** for 15 min. Polyethylene glycol [PEG, average molecular weight (MW) 8000] and NaCl was added to the supernatant to a final concentration of 7·5% w/v and 0·5 M, respectively. Virus was allowed to precipitate for 4 h at 4°C. Precipitated virus was pelleted at 10 000 × ***g*** for 30 min in a Sorvall centrifuge and suspended in 5 ml of TNE (10 mM Tris–HCl, pH 8·0, 100 mM NaCl, and 1 mM EDTA, pH 8·0). Suspended virus was then concentrated by ultracentrifugation in a SW41 rotor at 38 000 rpm through a 20–60% (w/w) sucrose gradient in TNE.[14] For CCHF virus, the supernatant material was precipitated with 8% (w/v) PEG containing 2·3% (w/v) NaCl for 3 h at 4°C. The precipitate was pelleted, resuspended in TNE, and overlayed onto a 15–65% (w/w) sucrose gradient in TNE. The gradients were centrifuged in a SW41 rotor at 40 000 rpm for 1·5 h at 4°C. Direct analysis of 1 ml fractions of the gradient, by SDS polyacrylamide gel electrophoresis, as described previously,[15] were performed. For Lassa virus

Table 1. Marburg primer sets

Primer set	Target gene	Amplicon length (bp)	TaqMan® assay	Reference
REM2/REM3*	NP	223	No	(Not published**)
FILOA/FILOB*	L	419	No	(Sanchez *et al.*[9])
MBGGP3	GP	143	Yes	(This study)

Key: * = Previously published primer sets. ** = Personal communication, Sanchez, Centers for Disease Control. Yes = TaqMan assay; No = Conventional RT-PCR assay. L = RNA-dependent RNA polymerase; NP = Nucleoprotein gene; GP = Glycoprotein gene.

purification, supernatant was clarified in a Sorvall centrifuge at 8000 rpm in a GSA rotor. Seven percent (w/v) PEG (average MW 6000) and 2·3% (w/v) NaCl was added to supernatant. The virus was allowed to precipitate for 3 h at 4 °C. The precipitate was then purified through a discontinuous 10–40% renograffin gradient (Bracco Diagnostics Inc., Princeton, NJ, USA) in a SW-41 rotor at 37 000 rpm for 75 min. The visible virus band was harvested and diluted in TNE. The diluted virus was then purified though a continuous 10–50% renograffin gradient at 30 000 rpm overnight. The virus was harvested, pelleted by centrifugation in a SW41 rotor at 38 000 rpm for 45 min and suspended in 2 ml of TNE (Jahrling, unpublished protocol). For VSV purification, cell culture supernatants were clarified by centrifugation at $10000 \times \boldsymbol{g}$ for 30 min. PEG (avg. MW 8000) and NaCl were added to clarified supernatant to 7 and 2·3% respectively. Virus was precipitated overnight at 4°C. Precipitated virus was then pelleted by centrifugation at $10\,000 \times \boldsymbol{g}$ for 30 min, resuspended in Dulbecco's phosphate-buffered saline (PBS), and then purified by centrifugation through a 20–60% (w/w) sucrose density gradient for 3·5 h at $100\,000 \times \boldsymbol{g}$. Sucrose solutions were prepared in Hank's balanced salt solution (HBSS) without $NaHCO_3$ containing 0·02 M*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 7·2).[16]

Filoviruses were titred by performing plaque assays on Vero E6 cells as described previously.[17] Viral stock was serially diluted in minimum essential medium with Earle salts (EMEM) containing non-essential amino acids (NEAA), adsorbed onto confluent Vero E6 cells in 12-well dishes, incubated for 1 h at 37°C, and covered with an agarose overlay. A 1:5000 dilution of neutral red in buffered saline solution was added 7 days later, and plaques were counted the following day.

Primer and probe design

The characteristics of selected primer and probe sets are listed in Table 1. Sequences of the Musoke, Ravn, and Popp strains of Marburg were aligned by using the DNA Star MegAlign software. After careful study of the aligned sequences, a highly conserved region of the *GP* gene was targeted for primer/probe development. A primer/probe set, designated MBGGP3, was designed by evaluating the Marburg virus glycoprotein gene [GenBank Accession Nos, Z29337 (Marburg, Popp strain); Z12132 (Marburg, Musoke strain); and AF005734 (Marburg, Ravn strain)] using Primer Express Software™ (Applied Biosystems). The primer and probe set was designed to meet the guidelines recommended by Applied Biosystems. In brief, the primers were designed with melting temperatures (T_m) between 58–60°C. Probes were designed to have a T_m at least 7–10°C higher than the primer T_m. The presence of a guanine at the 5′ end of probes was avoided and the amplicons were designed to be less than 200 bp in length. Primer and probe dimers, hairpins, stem-loops, and false-priming sites were minimized. The sequences of the primers are: MBGGP3 forward primer: 5′-TTCCCCTTTGGAGGCATC-3′; and MBGGP3 reverse primer: 5′-GGAGGATCCAACAGCAAGG-3′. The probe sequence is: MBGGP3Prb 5′-CGATGGGCTTTCAGGACAGGTGT-3′.

Fluorogenic 5′-nuclease assays

RT-PCRs were performed on an ABI PRISM™ 7700 Sequence Detection System (Applied Biosystems) using Applied Biosystems TaqMan® EZ RT-PCR kit in accordance with the manufacturer's instructions. Final concentrations used in the 50 µl reaction mix were: 0·5 µM of each primer, 0·2 µM of probe, 5 *U* r*Tth* enzyme, 300 µM deoxynucleotide triphosphates, 1 × Applied Biosystems TaqMan® RT-PCR buffer containing the passive reference dye ROX, and 2·5 mM manganese acetate. Thermocycling conditions for the MBGGP3 primer/probe set were as follows: 55°C for 45 min, 94°C for 1 min, followed by 40 cycles at 94°C for 15 s, and 60°C for 30 s. Total assay time on the ABI PRISM™ 7700 Sequence Detection System was approximately 2 h.

Conventional RT-PCR assays

Previously published primer sets (Table 1) were tested using the Applied Biosystems EZ r*Tth* RNA PCR kit (Applied Biosystems) according to the manufacturer's instructions. Thermocycling conditions for each set were published previously.[9] Final concentrations used in the 50 µl reaction mix were: 0·5 µM of each primer, 5 *U* r*Tth* enzyme, 300 µM deoxynucleotide triphosphates, 1 × Applied Biosystems EZ buffer, and 2·5 mM manganese acetate. All assays were performed on a PTC 100 thermocycler (MJ Research, Waltham, MA, USA).

Sequencing of RT-PCR products

To verify identity of PCR products, we sequenced amplicons from RT-PCR reactions using dye-labelled terminators and cycle sequencing (Taq Prism kit, Applied Biosystems). Products from sequencing reactions were analysed on an ABI 377 sequencer (Applied Biosystems). The sequences were then identified using the National Center for Biotechnology Information's Basic Local Alignment Search Tool (BLAST) (Internet web address: http://www.ncbi.nlm.nih.gov/BLAST/).

Limit of detection (LOD) assays

To determine plaque-forming unit (pfu) LODs, serial 10-fold dilutions of sucrose-purified Marburg virus stocks (Musoke strain and Ravn strain) were prepared in 1 ml of EMEM/NEAA. No sample controls were prepared using virus free EMEM/NEAA. One hundred microliters of each dilution were placed in Trizol LS (Gibco BRL, Gaithersburg, MD, USA), and RNA isolation was performed in accordance with the manufacturer's instructions. Briefly, chloroform was added to the virus/Trizol LS mixture and following centrifugation the aqueous phase containing the RNA was harvested. RNA was then precipitated with the addition of isopropanol, washed with 75% ethanol, and resuspended in 100 µl of RNase free water. One microliter of each RNA preparation was used in subsequent TaqMan® and conventional RT-PCR assays. TaqMan® assays were performed in triplicate. In order to quantify the virus titre of the purified virus preparations, serial 10-fold dilutions were plaqued on Vero E6 cells as previously described (see above). The LOD was defined as the last dilution in the fluorogenic 5′-nuclease assays in which the C_T value (threshold cycle) was less than 40.

To determine the LODs of purified viral RNA, sucrose purified virus was placed in Trizol LS (Gibco BRL), and RNA isolation was performed in accordance with the manufacturer's instructions. Briefly, chloroform was added to the virus/Trizol LS mixture, centrifuged, and the aqueous phase containing the RNA was harvested. Viral RNA was then precipitated with the addition of isopropanol, washed with 75% ethanol, and resuspended in RNase free water. The RNA was quantitated by spectrophotometry and serial 10-fold dilutions were prepared from 1 ng to 10 fg μl^{-1}. One microliter of each dilution was used in subsequent TaqMan® and conventional RT-PCR assays. The LODs of the newly designed TaqMan® assay were compared to the LODs of previously designed Marburg primers (Table 2).[9]

Blind tissue panel

Spleen, brain, lung, liver, and kidney were collected from non-human primates that died following a challenge with Marburg virus (Ravn strain). All tissues were shown to be positive for Marburg virus in subsequent plaque assays (Hevey, personal communication). In parallel, control tissues were collected from healthy non-human primates obtained commercially (BioWhittaker, Walkersville, MD, USA). Tissues were snap frozen in liquid nitrogen and stored at −70°C until processing. For RNA extraction, tissues were weighed, placed in the appropriate amount (1 ml per 100 mg of tissue) of Trizol (Gibco BRL), and homogenized. The RNA was extracted as described above. Five microliters of each 100 µl RNA preparation was used in subsequent TaqMan® assays. The study was blinded by an independent investigator who assigned random numbers to each sample.

Cross-reactivity panel

To evaluate the cross-reactivity of the MBGGP3 primer/probe set, RNA from genetically related viruses [vesicular stomatitis virus, measles virus, Ebola virus (Zaire, Sudan, Ivory Coast, Reston strains)] and other haemorrhagic fever viruses (Lassa virus and Crimean-Congo haemorrhagic fever virus) was isolated. The RNA from these viruses was extracted from purified viral seeds with Trizol LS as described previously. One nanogram of each viral template was used for cross-reactivity tests. The cross-reactivity panel was blinded by an independent investigator who assigned random numbers to each sample.

Table 2. Sensitivity of Marburg virus primer/probe sets

Primer Set	Musoke Limit of detection (LOD)		RAVN-LOD	
	pfu	Mass	pfu	Mass
REM2/REM3*	5 pfu	10 pg	300 pfu	1 ng
FILOA/FILOB*	80 pfu	10 pg	Not detected	
MBGGP3	5 pfu	1 pg	2 pfu	10 pg

Key: * Previously designed primer sets. pfu = plaque forming units. PFU LODs are based on a known number of plaque forming units from which viral RNA was extracted. Mass LODs are based on a known amount of purified viral RNA.

Direct analysis and detection of RT-PCR-amplified products on agarose gels

Ten µl aliquots of the RT-PCR products were electrophoresed on 4% agarose gels (FMC BioProducts, Rockland, ME, USA) in Tris–borate buffer (TBE) containing 1 µg per ml ethidium bromide. DNA bands were visualized with an Alpha-Inotech imaging system (Alpha Inotech Corporation, San Leandro, CA, USA).

RESULTS

TaqMan® primer/probe design

Two hundred possible sets of primers and probes specific for one of the highly conserved regions of the Marburg GP gene were generated using the Primer Express™ software. The MBGGP3 primer/probe set was chosen based on guidelines recommended by PE Applied Biosystems, as outlined previously in the materials and methods. The primer/probe set chosen, MBGGP3, was designed to amplify a 143 bp region of the *GP* gene.

Limit of detection experiments

The MBGGP3 primer/probe set limit of detection was equivalent to or 10–100 times greater than previously designed primer sets intended for use in conventional RT-PCR assays (Table 2). The MBGGP3 primer/probe set was able to detect 1 pg of purified Marburg (Musoke strain) RNA and 5 pfu of infectious virus (Table 2 and Fig. 1a). MBGGP3 was also able to detect 10 pg of purified Marburg (Ravn strain) RNA and 2 pfu infectious virus (Table 2 and Fig. 1b). Previously designed primer sets (REM2/REM3 and FILOA/FILOB) for use in conventional RT-PCR assays were able to detect 10 pg and 5 pfu (REM2/REM3) or 10 pg and 80 pfu (FILOA/FILOB) of purified Marburg (Musoke strain) RNA or infectious virus, respectively. REM2/REM3 was able to detect 1 ng of Marburg (Ravn strain) RNA and 300 pfu infectious virus. The FILOA/FILOB primer set was not able to detect Marburg virus (Ravn strain) (Table 2). All no sample controls were negative. TaqMan® results were confirmed by observing the expected band size on 4% agarose gels.

Cross-reactivity experiments

Several genetically related viruses and other haemorrhagic fever viruses were used to determine the cross-reactivity of the MBGGP3 primer/probe set. The MBGGP3 primer/probe set detected both strains of Marburg virus tested (Musoke and Ravn) but did not detect any of the genetically related or other haemorrhagic fever viruses (Table 3). Although REM2/REM3 detected both the Musoke and Ravn strains of Marburg virus, it also amplified Ebola Zaire. The FILOA/FILOB primer set was able to detect Marburg virus (Musoke strain), however, it also amplified Ebola viruses [Zaire, Sudan, and Ivory Coast (IC) strains]. The amplicon observed with FILOA/FILOB and 1 ng of Ebola IC template was very faint. Therefore, neither of the previously designed primer sets (REM2/REM3 or FILOA/FILOB) was specific for Marburg virus (Table 3). TaqMan® results were confirmed by observing the expected band size on 4% agarose gels.

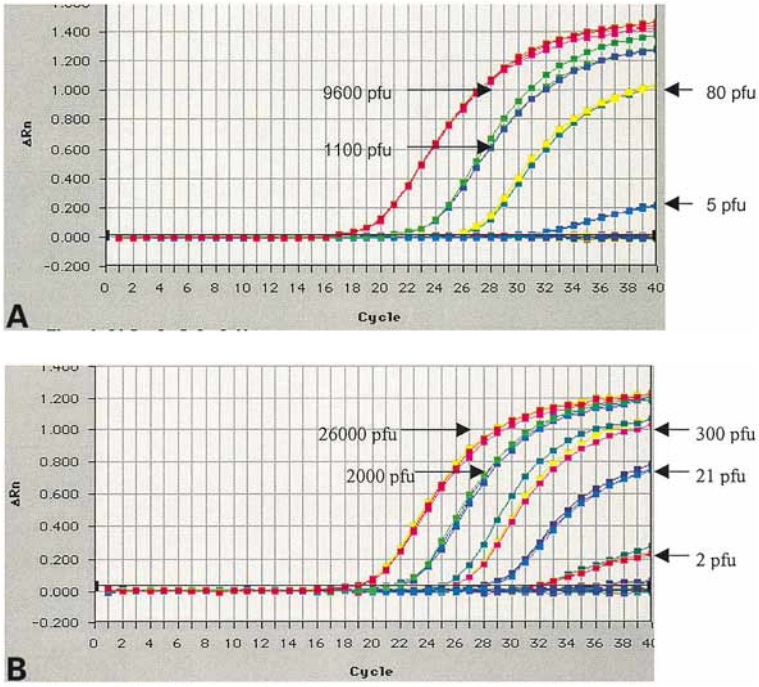

Fig. 1. Sensitivity of MBGGP3 TaqMan® Assay for (a) Marburg virus (Musoke strain) and (b) Marburg virus (Ravn strain). Total RNA isolated from serial dilutions of sucrose-purified virus was used as the template. Each sample was run in triplicate. The graphs depict amplification plots for both templates.

Table 3. Cross reactivity panel: Marburg virus primer sets

Primer Set	Lassa	Measles	VSV	CCHF	Ebo-Z	Ebo-S	Ebo-IC	Ebo-R	MBG-RAVN	MBG-MSK
MBGGP3	−	−	−	−	−	−	−	−	+	+
REM2/REM3*	ND	ND	ND	ND	+	−	−	−	+	+
FILOA/FILOB*	ND	ND	ND	ND	+	+	+	−	−	+

Key: * Previously designed primer sets. VSV (vesicular stomatitis virus); CCHF (Crimean-Congo haemorrhagic fever virus); Ebo-Z (Ebola-Zaire); Ebo-S (Ebola-Sudan); Ebo-IC (Ebola-Ivory Coast); Ebo-R (Ebola-Reston); MBG-RAVN (Marburg Ravn); MBG-MSK (Marburg Musoke). + = amplicon detected, − = no amplicon detected, ND = No data.

Detection of Marburg Ravn from infected cynomolgus monkeys

Specificity of the MBGGP3 primer/probe set was tested in a blinded fashion with RNA isolated from 20 control tissues and 10 tissues harvested from non-human primates infected with Marburg virus (Ravn strain). The clinical specificity of MBGGP3 primer/probe set was 100% (Table 4). Fluorogenic 5′-nuclease assays performed with viral RNA from infected tissues produced C_T values of <40, indicating the sample was positive for Marburg virus, and also resulted in the expected 143 bp amplicon when observed by gel electrophoresis. In contrast, control samples did not cross threshold during the 40 cycles of the PCR assay (indicating an absence of Marburg virus) and no amplicons were observed by gel electrophoresis in these samples.

Table 4. Blind tissue panel: Marburg Ravn infected non-human primates

True positives	False positives	True negatives	False negatives
10	0	20	0

Specificity of MBGGP3 primer-probe set tested in a blind tissue study. A blind tissue study was performed with tissues obtained from a live non-human primate challenge with Marburg virus (Ravn strain). Specificity of the newly designed primer/probe set was determined in a TaqMa^n® assay. Uninfected non-human primate tissues served as controls. (n = 30)

DISCUSSION AND CONCLUSIONS

This report describes the development and evaluation of a one-tube fluorogenic RT-PCR assay on the ABI PRISM™ 7700 Sequence Detection System for the

identification of Marburg virus infections. The *GP* gene of Marburg virus was chosen as an assay target for several reasons. First, the GP protein of Marburg and Ebola viruses is required for virus entry into cells. Second, the membrane-bound GP of Ebola virus is reported to play a central role in the virus-mediated cytotoxicity of endothelial cells and therefore appears to be an important virulence factor of the Ebola virus.[18] Finally, the *GP* gene of all Marburg virus strains contains highly conserved regions that permitted the design of primer/probe sets based on the guidelines set forth by Applied Biosystems.

This assay has several advantages over conventional RT-PCR assays that are currently used for the identification of Marburg virus. The LOD of this assay was equivalent to or 10–100-fold greater than that of previously designed primer sets (Table 2). REM2/REM3 was capable of detecting both Marburg virus strains tested (Musoke and Ravn), but only at a limit of 10 pg and 1 ng, respectively. FILOA/FILOB was only capable of amplifying the Musoke strain. Based on LOD assays, the MBGGP3 primer/probe set was capable of detecting 1 pg of Marburg virus (Musoke strain) and 10 pg of Marburg virus (Ravn strain) purified nucleic acids (Table 2). In order to express the limit of detection of MBGGP3 primer/probe set as a more clinically relevant number, LOD assays were performed on serial dilutions of purified virus containing a known amount of pfu. The MBGGP3 primer/probe set was capable of detecting 5 pfu of Marburg virus (Musoke strain) and 2 pfu of Marburg virus (Ravn strain). The discrepancy between the pfu LODs and the mass LODs (expressed as the total amount of viral RNA added to assay) can be explained by the method by which each assay was performed and quantitated. The mass LOD assays were performed by preparing serial 10-fold dilutions of an initial stock of RNA that was quantitated by spectrophotometry. The LOD assays expressed in total pfu were performed by serially diluting sucrose-purified viral stocks and then determining the titres of each dilution. Therefore, with the plaque assay method for determining LOD, the number of pfu were determined after the dilutions were prepared. With the mass LOD method, the amount of RNA in each dilution was not determined subsequent to dilutions being prepared. Therefore, it is likely that the LODs expressed in pfu are in fact more accurate than the LOD assays performed with total RNA alone (mass LODs).

The MBGGP3 assay was highly specific for Marburg virus. Other primer sets used in conventional RT-PCR assays did not show the same level of specificity. The MBGGP3 primer/probe set was specific for all Marburg strains tested in a blinded cross-reactivity panel and showed an estimated clinical specificity of 100% when tested against a blind tissue panel (Table 4). The blinded tissue panel contained tissues from non-human primates challenged with Marburg virus (Ravn strain). In contrast, previously designed primer/probe sets amplified one (REM2/REM3) or more (FILOA/FILOB) subtypes of Ebola virus and therefore lacked the specificity of the MBGGP3 primer/probe set (Table 3).

The one-tube fluorogenic RT-PCR assay is compatible with emerging rapid nucleic-acid analysis platforms.[19] These new platforms are capable of performing both thermocycling and fluorescence detection in real time. Sources of these instruments include Applied Biosystems, Roche Diagnostics (Indianapolis, IN, USA), Idaho Technologies (Salt Lake City, UT, USA), Cepheid (Sunnyvale, CA, USA) and Bio-Rad (Hercules, CA, USA). With the more rapid thermocycling instruments, [Lightcycler™ (Roche Molecular Systems), the ruggedized Advanced Pathogen Identification Device (R.A.P.I.D.™, Idaho Technologies) or the SmartCycler™ (Cepheid)], it is reasonable to assume that once a sample is received and processed, a definitive diagnosis can be made within 20–40 min.[20] In addition, the use of this technology in field laboratories has become a reality with availability of instruments such as the R.A.P.I.D.™ and SmartCycler XC™, which have been specifically designed for use by field medical laboratories.

To obtain an early diagnosis and contain future outbreaks of Marburg haemorrhagic fever, it will be necessary to implement a rapid diagnostic assay in laboratories throughout Africa. This assay must not require great levels of technical skill to perform and must be accurate and reproducible. Transfer of the newly designed fluorogenic 5′ nuclease assay described in this study to rapid nucleic-acid analysis platforms would be a first step towards providing public health authorities in Africa with a tool to help diagnose and contain possible outbreaks. Future developments producing a dried-down chemistry for RT-PCR (making the addition of water and sample the only reagents needed for performing the fluorogenic RT-PCR assay) would make this assay more easy to use in the field and provide a valuable diagnostic capability that would enhance future outbreak investigations.

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