Am. J. Trop. Med. Hyg., 82(5), 2010, pp. 954–960
doi:10.4269/ajtmh.2010.09-0636
Copyright © 2010 by The American Society of Tropical Medicine and Hygiene

Short Report: Comprehensive Panel of Real-Time TaqMan™ Polymerase Chain Reaction Assays for Detection and Absolute Quantification of Filoviruses, Arenaviruses, and New World Hantaviruses

Adrienne R. Trombley, Leslie Wachter, Jeffrey Garrison, Valerie A. Buckley-Beason, Jordan Jahrling, Lisa E. Hensley, Randal J. Schoepp, David A. Norwood, Augustine Goba, Joseph N. Fair, and David A. Kulesh*

Diagnostic Systems Division, and Virology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland; Medimmune, Gaithersburg, Maryland; Battelle, Columbus, Ohio; Department of Neuroscience, University of Texas Medical Branch, Galveston, Texas; The Global Viral Forecasting Initiative, San Francisco, California; Lassa Fever Laboratory, Kenema Government Hospital, Kenema, Sierra Leone

Abstract. Viral hemorrhagic fever is caused by a diverse group of single-stranded, negative-sense or positive-sense RNA viruses belonging to the families *Filoviridae* (Ebola and Marburg), *Arenaviridae* (Lassa, Junin, Machupo, Sabia, and Guanarito), and *Bunyaviridae* (hantavirus). Disease characteristics in these families mark each with the potential to be used as a biological threat agent. Because other diseases have similar clinical symptoms, specific laboratory diagnostic tests are necessary to provide the differential diagnosis during outbreaks and for instituting acceptable quarantine procedures. We designed 48 TaqMan™-based polymerase chain reaction (PCR) assays for specific and absolute quantitative detection of multiple hemorrhagic fever viruses. Forty-six assays were determined to be virus-specific, and two were designated as pan assays for Marburg virus. The limit of detection for the assays ranged from 10 to 0.001 plaque-forming units (PFU)/PCR. Although these real-time hemorrhagic fever virus assays are qualitative (presence of target), they are also quantitative (measure a single DNA/RNA target sequence in an unknown sample and express the final results as an absolute value (e.g., viral load, PFUs, or copies/mL) on the basis of concentration of standard samples and can be used in viral load, vaccine, and antiviral drug studies.

The disease-causing hemorrhagic fever RNA viruses have the potential to be used as aerobiological weapons, indicating that accurate and timely identification of these agents is necessary.[1,2] The implicated hemorrhagic fever viruses include Ebola, Marburg, Lassa, Junin, Machupo, Sabia, Guanarito, and hantavirus. TaqMan™-based, real-time reverse transcription–polymerase chain reaction (RT-PCR) can rapidly detect and identify the selected gene targets found within the genomes of these RNA viruses. Although many research groups have developed and tested standard and real-time RT-PCR assays for detection of various species and corresponding strains of Ebola,[3–8] Marburg,[4,8,9] Lassa[8,10–12] Junin,[13] and hantavirus,[14–18] a comprehensive panel of TaqMan™-based real-time PCR assays using identical cycling conditions is not currently available.

We designed 48 TaqMan™-based PCR assays for specific and absolute quantitative detection of multiple hemorrhagic fever viruses. A select few of these assays were used with unidentified human isolates drawn from suspected Lassa fever cases at the Kenema Government Hospital in Sierra Leone. The approved human use protocols for this study are United States Army Medical Research Institute of Infectious Diseases FY09-32 and Tulane University Institutional Review Board Study #09-00332.

Primer pairs and TaqMan™/TaqMan™-minor groove binder (MGB) probes were designed with either Primer Express version 2.0 (PE2) (Applied Biosystems, Foster City, CA; www.appliedbiosystems.com) or AlleleID 4/5/6 (PREMIER BioSoft, Palo Alto, CA; www.premierbiosoft.com) using gene targets and genomes identified by accession numbers in Table 1. Hemorrhagic fever virus family genomes were initially aligned using ClustalW2 (http://www.ebi.ac.uk/clustalw/index.html) with nonhomologous regions identified visually. The PE2 software was then implemented to design virus-specific TaqMan™ and TaqMan™-MGB assays. Viral genomes aligned with AlleleID used the integrated ClustalW2 algorithms with the Species-Specific Design option selected in the software. After primer/probe targets were chosen by the software, corresponding viral genes were identified. Development of pan assays, defined as a single assay capable of detecting multiple viral sequences, were also attempted using the Taxa-Specific/Cross-Species Design option of AlleleID. AlleleID was able to design pan assays for detection of Marburg virus only (Table 1). The design of virus-specific assays by both software programs resulted in 48 primer/probe pairs (27 TaqMan™-MGB and 21 TaqMan™) using several gene targets: nucleoprotein, glycoprotein, virus protein 40, polymerase, and zinc-binding protein.

Virus preparations were produced in continuous cell lines appropriate for each virus. Virus stocks were harvested prior to peak cytopathic effects to minimize contaminating cellular components such as RNA or DNA. The cell culture supernatant containing intact virions was clarified by high-speed centrifugation. For extraction of viral RNA, virus stock was added to a virus-inactivating volume (3:1) of TRIzol reagent (Invitrogen, Grand Island, NY; www.invitrogen.com). Four-hundred microliters of TRIzol-inactivated virus was either extracted manually using the Ambion MagMAX™ Viral RNA Isolation Kit (Ambion, Austin, TX; www.ambion.com) or automated using the BioRobot EZ1 Virus Mini Kit V 2.0 (Qiagen, Valencia, CA; www.qiagen.com). All viral genomic RNAs were eluted in 60 μL of elution buffer (Ambion) or AVE buffer (Qiagen) and stored at −80°C. In a related study, viral RNA extracted by the Qiagen and Ambion methods was directly compared using identical assay conditions and viral genomic RNA concentrations and resulted in uniform cycle threshold (C_t) regardless of extraction method.[19]

All real-time RT-PCR assays were performed with specific primers and probes using the Invitrogen SuperScript™

*Address correspondence to David A. Kulesh, Diagnostic Systems Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD 21701-5011. E-mail: David.Kulesh@us.army.mil

TABLE 1
Real-time PCR assay primers, probes, reaction concentrations and sensitivities*

RNA viruses	Target	Genome accession #	Amplicon size	Primers/probe	Sequence (5′ - 3′)	Final conc (μM)	Sensitivity (PFU/PCR)†
Ebola Zaire-MGB	NP	AF086833	76	F565 R640 p597S	5′ - TCT GAC ATG GAT TAC CAC AAG ATC - 3′ 5′ - GGA TGA CTC TTT GCC GAA CAA TC - 3′ 6FAM - AGG TCT GTC CGT TCA A - MGBNFQ	0.9 0.9 0.2	0.001
Ebola Sudan-MGB	NP	AY729654	80	F1051 R1130 p1079S	5′ - CAT GCA GAA CAA GGG CTC ATT C - 3′ 5′ - CTC ATC AAA CGG AAG ATC ACC ATC - 3′ 6FAM - CAA CTT CCT GGC AAT - MGBNFQ	1.0 1.0 0.2	0.1
Ebola Reston-MGB	GP	AB050936	55	F1129 R1183 p1149S	5′ - TCA CCG CGA ACC CAA TG - 3′ 5′ - TCG CTT GTC ATG GTT GGA CTT - 3′ 6FAM - ACC ACC ATT GCC C- MGBNFQ	0.8 0.8 0.2	1.0 (34 copies)‡
Ebola Ivory Coast-MGB	GP	U28006	64	F1123 R1186 p1143S	5′ - CCC ATC TCC GCC CAC AA - 3′ 5′ - GAG TGG AAT CCT CTG AAA CCA ATT - 3′ 6FAM - CGC AGG CGA AGA C - MGBNFQ	0.7 0.7 0.2	1.0 (586 copies)‡
Ebola Zaire-TM	GP	AF086833	80	F2000 R2079 p2058A	5′ - TTT TCA ATC CTC AAC CGT AAG GC - 3′ 5′ - CAG TCC GGT CCC AGA ATG TG - 3′ 6FAM - CAT GTG CCG CCC CAT CGC TGC - TAMRA - 3′	1.0 1.0 0.1	0.0001 (584 copies)‡
Ebola Sudan-TM	GP	AY729654	77	F583 R659 p608SB	5′ - AGG ATG GAG CTT TCT TCC TCT ATG - 3′ 5′ - TAC CCC CTC AGC AAA ATT GAC T - 3′ 6FAM - CAG GCT GGC TTC AAC TGT AAT TTA CAG AGG - TAMRA -3′	0.8 0.8 0.1	0.1
Ebola Reston-TM	VP40	AF522874	80	F5645 R5724 p5674S	5′ - CTA TGG TTA TCA CCC AGG ATT GTG - 3′ 5′ - GTA ACT ATC CTG CTT GTC CAT GTG - 3′ 6FAM - TGC CAC TCT CCA GCC AGC CAT CCG - TAMRA - 3′	0.9 0.9 0.1	1.0
Ebola Ivory Coast-TM	GP	U28006	79	F564 R642 p589S	5′ - TGT ACA CAA AGT CTC AGG AAC TGG - 3′ 5′ - GTC ATA CAG GAA GAA GGC TCC TTC - 3′ 6FAM - CCA TGC CCA GGA GGA CTC GCC TTT - TAMRA -3′	1.0 1.0 0.1	0.1
Ebola Bundibugyo (MGB and TM)	NP	FJ217161	74	F2016 R2089 p2045S p2038S	5′ - ATG GAA ACC AAG GCG AAA CTG - 3′ 5′ - TAC TTG TGG CAT TGG CTT GTC T - 3′ 6FAM - CGG GTA GCC CCC AAC - MGBNFQ or 6FAM - ATC CCC ACG GGT AGC CCC CA - TAMRA	0.9 0.9 0.2 0.1	10^{-6} (RNA dilution)§ (MGB and TM)
***pan*-Marburg-MGB assay:** (Ravn, Ci67, Musoke, and Angola-MGB)	GP	EF446131 (Ravn) EF446132 (Ci67) DQ217792 (Musoke) DQ447660 (Angola)	64	F6121 F6121-1 R6184 p6144	5′ - GAT TCC CCT TTG GAA GCA TCT - 3′ 5′ - GAT TCC CCT TTA GAG GCA TCC - 3′ 5′ - CAA CGT TCT TGG GAG GAA CAC - 3′ 6′FAM - ACG ATG GGC TTT CAG - MGBNFQ - 3′	1.0 1.5 1.0 0.2	0.1 (Ravn) 1.0 (Ci67) 10 (Musoke) 1.0 (Angola)
***pan*-Marburg-TM assay:** (Ravn, Ci67, Musoke, and Angola-TM)	GP	EF446131 (Ravn) EF446132 (Ci67) DQ217792 (Musoke) DQ447660 (Angola)	64	F6121 F6121-1 R6184 p6145A-5 p6145A-7	5′ - GAT TCC CCT TTG GAA GCA TCT - 3′ 5′ - GAT TCC CCT TTA GAG GCA TCC - 3′ 5′ - CAA CGT TCT TGG GAG GAA CAC - 3′ 6FAM - AAA CGA TGG GCC TTC AGG GCA GG-TAMRA-3′ 6FAM - AAG CGA TGG GCT TTC AGG ACA GG-TAMRA-3′	0.5 1.0 1.0 0.1 0.1	0.1 (Ravn) 10 (Ci67) 1.0 Musoke) 10 (Angola)

(*continued*)

TABLE 1
Continued

RNA viruses	Target	Genome accession #	Amplicon size	Primers/probe	Sequence (5′ - 3′)	Final conc (μM)	Sensitivity (PFU/PCR)†
Marburg Musoke-MGB	NP	DQ217792	65	F391 R455-3 p429A	5′ - CAA CCC GCT TTC TGG ATG TG - 3′ 5′ - CTT AAG GGC TAG AAT TAA AGG GCT - 3′ 6FAM - TAA TGA GGT TCG TTA GGA A - MGBNFQ	1.0 1.0 0.2	10
Marburg Ci67-MGB	NP	EF446132	65	F391 R455 p413S	5′ - CAA CCC GCT TTC TGG ATG TG - 3′ 5′ - CTT AAG GGC CAA AAT TAA AGG ACT G -3′ 5′ - 6FAM - TCC TAA CGA ACC TCA - MGBNFQ	0.9 0.9 0.2	0.01 (120 copies)‡
Marburg Ravn-MGB	NP	EF446131	66	F350 R415 p371S	5′ - GGA CGC GGG CTA TGA GTT TG - 3′ 5′ - GGA ATA ACC TCT AGA AAG CGA GTT G -3′ 6FAM - TGT CAT CAA GAA TCC TG - MGBNFQ	0.9 0.9 0.2	0.1
Marburg Angola-MGB	VP40	DQ447660	66	F4573T R4638G p4601CT	5′ - CCA GTT CCA GCA ATT ACA ATA CAT ACA - 3′ 5′ - GCA CCG TGG TCA GCA TAA GGA - 3′ 6FAM - CAA TAC CTT AAC CCC C - MGBNFQ	0.6 0.6 0.2	0.1
Marburg Ravn-TM	NP	DQ447649	77	F1788 R1864 p1815S	5′ - TTA TAT GCT CAG GAA AAG AGA CAG G - 3′ 5′ - CCA ATA CTG CCA AAG GGA TCT TG - 3′ 6FAM - CCC ATA CAG CAT CCA GCC GTG AGC - TAMRA - 3′	0.9 0.9 0.1	0.1
Marburg Angola-TM	NP	DQ447660	80	F985 R1064 p1035A	5′ - TCT ATC CTC AGC TCT CAG CAA TTG - 3′ 5′ - TTC GCC GAC ATT GAC ACC AG - 3′ 5′ - 6FAM - TGC CAT GTG CTG TCG CTA CAC CCA - TAMRA - 3′	1.0 1.0 0.1	0.1
Lassa Josiah-MGB	GPC	AY628203	80	F3569 R3648 p3598S	5′ - TGC TAG TAC AGA CAG TGC AAT GAG - 3′ 5′ - TAG TGA CAT TCT TCC AGG AAG TGC - 3′ 6FAM - TGT TCA TCA CCT CTT C - MGBNFQ	0.9 0.9 0.2	0.1 (268 copies)‡
Lassa Macenta-MGB	NP	AY628201	73	F1079 R1151 p1106S	5′ - CAG GAA GGG CAT GGG AAA - 3′ 5′ - TTG TTG CTC CCA ATT TTT TGT G - 3′ 6FAM - TTG ATT TGG AAT CAG GCG AG - MGBNFQ	0.8 0.8 0.2	10^{-6} (RNA dilution)§ (257 copies)‡
Lassa Weller-MGB	NP	AY628206	72	F1695 R1766 p1720S	5′ - GCA TTG ATG GAC TGC ATT ATG TTT - 3′ 5′ - CAC AGC TCT TAG GAC CTT TGC AT - 3′ 6FAM - ATG CAG CAG TCT CGG GA - MGBNFQ	0.9 0.9 0.2	0.1 (583 copies)‡
Lassa Pinneo-MGB	GPC	AY628207	78	F3525 R3602 p3559S	5′ - CCA ATA ATC CCA CAT GTA GCG ATG - 3′ 5′ - GAA CAT TGT GCT AAT TGC GCT TTC - 3′ 6FAM - CCT TCA AGA TTG CCA - MGBNFQ	0.9 0.9 0.2	0.001
Lassa Mozambique (Mopeia)-MGB	NP	DQ328874	78	F1788 R1865 p1815S	5′ - TCT GGG GAC CGG CAA TTG TG - 3′ 5′ - ACA CCA CAT TGT GCC TTA CTA GAC - 3′ 6FAM - TAT GAC TGC TGC TTC - MGBNFQ	0.9 0.9 0.2	1.0
Lassa Mobala (Acar)-MGB	GPC	AY342390	80	F1858 R1937 p1915A	5′ - TAC AGA CCA CAG CTA CAC ACA CC - 3′ 5′ - ACT CAC CGT CAC CTG GTT GG - 3′ 6FAM - AGC CGT GCC CAA AG - MGBNFQ	1.0 1.0 0.2	0.001
Lassa Josiah-TM	NP	AY628203	70	F548 R617 p594A	5′ - GGA ATG AGT GGT GGT AAT CAA GG - 3′ 5′ - TTT TCA CAT CCC AAA CTC TCA CC - 3′ 6FAM - ACT CCA TCT CTC CCA GCC CGA GC-TAMRA - 3′	0.6 0.6 0.1	0.1 (11,711 copies)‡

(*continued*)

Table 1
Continued

RNA viruses	Target	Genome accession #	Amplicon size	Primers/probe	Sequence (5′ - 3′)	Final conc (μM)	Sensitivity (PFU/PCR)†
Lassa Macenta-TM	NP	AY628201	73	F1079 R1151 p1098S	5′ - CAG GAA GGG CAT GGG AAA - 3′ 5′ - TTG TTG CTC CCA ATT TTT TGT G - 3′ 6FAM – CAC TGT TGT TGA TTT GGA ATC AGG CGA GAA G - TAMRA - 3′	0.8 0.8 0.1	10^{-5} (RNA dilution)§ (257 copies)‡
Lassa Weller-TM	NP	AY628206	72	F1695 R1766 p1720S	5′ - GCA TTG ATG GAC TGC ATT ATG TTT - 3′ 5′ - CAC AGC TCT TAG GAC CTT TGC AT - 3′ 6FAM - ATG CAG CAG TCT CGG GAG GGC TC-TAMRA - 3′	0.9 0.9 0.1	0.01 (583 copies)‡
Lassa Pinneo-TM	GP	AY628207	81	F2730 R2810 p2757S	5′ - CCC AGT TTC CCT TTC CTG AGT - 3′ 5′ - CCA ACG GAG TGT TGC AAA CA - 3′ 6FAM - CAA TGT ATC TTC CAC CCC AGG CCA TTC - TAMRA - 3′	0.8 0.8 0.1	0.1 (234 copies)‡
Lassa Mozambique (Mopeia)-TM	NP	DQ328874	81	F2687 R2768 p2712S	5′ - CCT GAT GGT CTC CAG CAT ATT TC - 3′ 5′ - GCT ACA ATT TCA GCT TGT CTG C - 3′ 6FAM - CCC GTC TAT GAG GCA AGC CCC AGC - TAMRA - 3′	0.9 0.9 0.1	0.1
Lassa Mobala (Acar)-TM	GP	AY342390	85	F1860 R1944 p1915A	5′ - CAG ACC ACA GCT ACA CAC ACC - 3′ 5′ - AAT TCC AAC TCA CCG TCA CCT G - 3′ 6FAM - AGC CGT GCC CAA AGC CTC ATC GTC TC - TAMARA - 3′	1.0 1.0 0.1	0.0001
Machupo Carvallo-MGB	GPC	AY619643	80	F699 R778 p721S	5′ - ATG ACC CGT GTG AGG AAG GG - 3′ 5′ - GCC ACA GTA GTC AAA GGA ACT GG - 3′ 6FAM - AGT GTG CTA CCT GAC CAT - MGBNFQ - 3′	0.7 0.7 0.2	0.001
Machupo Mallele-MGB	GPC	AY619645	80	F406 R485 p428S	5′ - CAC CTT CCT GAT TCG GGT CTC - 3′ 5′ - CAA GGT CTT CTG GTT CAT AGA CTG - 3′ 6FAM - AGT GTA TCT GTC CTC A - MGBNFQ - 3′	0.8 0.8 0.2	0.001
Machupo Carvallo-TM	GPC	AY619643	78	F681 R758 p728A	5′ - ATC TCT TCA GGG GCT TCC ATG - 3′ 5′ - TGG GGT CAC CAC ACT GAT TG - 3′ 6FAM - AGC ACA CTT TCC CTT CCT CAC ACG G - TAMRA - 3′	0.7 0.7 0.1	0.001
Machupo Mallele-TM¶	Polymerase	AY619642	78	F1009 R1086 p1059A	5′ - TTC GAT CAA CAT TGC AGC TAA ATC - 3′ 5′ - GAT GGT GTA TCG GTT GTT GCA G- 3′ 6FAM - CCA TTG TTC ACC GGG CAG GCC AGT - TAMRA - 3′	0.5 0.5 0.1	0.001
Junin-MGB	NP	AY619641	65	F1933 R1997 p1956S	5′ - CAT GGA GGT CAA ACA GCT TCC T - 3′ 5′ - GCC TCC AGA CAT GGT TGT GA - 3′ 6FAM - ATG TCA TCG GAT CCT T - MGBNFQ	0.8 0.8 0.2	0.001 (234 copies)‡
Junin-MGB	Polymerase	AY619640	68	F2427 R2494 p2452S	5′ - TTG CCA AAT TGA CCC ATC TGT A - 3′ 5′ - GCA ATA TGC CGG GTG TAG TGA - 3′ 6FAM - TAG AGT TCG TCT GAT TTC A - MGBNFQ	0.8 0.8 0.2	0.001 (234 copies)‡
Junin-TM	NP	AY619641	79	F3032 R3110 p3083A	5′ - CAG TTC ATC CCT CCC CAG ATC - 3′ 5′ - GGT TGA CAG ACT TAT GTC CAT GAA GA - 3′ 6FAM - TGT TCA ACG AAA CAC AGT TTT CAA GGT GGG - TAMRA - 3′	1.0 1.0 0.1	0.0001

(*continued*)

Table 1
Continued

RNA viruses	Target	Genome accession #	Amplicon size	Primers/probe	Sequence (5′ - 3′)	Final conc (μM)	Sensitivity (PFU/PCR)†
Junin-TM	Zinc Binding Protein (Z)	AY619640	66	F282	5′ - AGG AAT TCG GAC CTC TGC AA - 3′	1.0	0.001
				R347	5′ - CTC CAC CGG CAC TGT GAT T - 3′	1.0	
				p303Sa	6FAM - ATC TGT TGG AAG CCC CTA CCT ACC A - TAMRA -3′	0.1	
Sabia-MGB	GP	U41071	65	F903	5′ - CGT CAG ATC TTA AGT GCT TTG GAA - 3′	0.8	0.001
				R967	5′ - TTC CGA ATC GTG GTC AAG GT - 3′	0.8	
				p928S	6FAM - CAC AGC ACT AGC AAA A - MGBNFQ	0.2	
Sabia-MGB	Polymerase	AY358026	73	F948	5′ - TGT GAT CAT TGG TCG CAG CTA - 3′	0.7	0.01
				R1020	5′ - CTG AGA GGG AAG AAG GCA GTG A - 3′	0.7	
				p970S	6FAM - ATC GTT ATC CAT TAG AAT CT - MGBNFQ	0.2	
Sabia-TM	GP	U41071	83	F903	5′ - CGT CAG ATC TTA AGT GCT TTG GAA - 3′	0.8	0.001
				R985	5′ - TTT CAA CAT GTC ACA GAA TTC CG - 3′	0.8	
				p959A	6FAM - CGT GGT CAA GGT TAC ATT TTG CTA GTG CTG TGT - TAMRA - 3′	0.1	
Sabia-TM	Hairpin Intergenic Region and RNA Polymerase	AY358026	79	F433	5′ - CCG GGG TGG TGT GGT TTA - 3′	0.7	0.01
				R511A	5′ - AAG GCT CAA GGG TGT TAC CTG - 3′	0.7	
				p460S	6FAM - TTC AAC GGA CTG CTG CTG TCT AAA TAG TCT - TAMRA - 3′	0.1	
Guanarito-MGB	GP1	AY129247	73	F485	5′ - TGG ATT CTT GGG TGG ACA ATT AA - 3′	0.6	0.01
				R557	5′ - TAG GCT CAC AGC AGA TTC TTG GA - 3′	0.6	
				p513S	6FAM - TGG GAC ATG ACT TTT T - MGBNFQ - 3′	0.2	
Guanarito-MGB	Polymerase	AY358024	80	F521	5′ - GCT GCC GGA GCT GTC TGA - 3′	0.7	0.1
				R599	5′ - ATG GTG CGA GTT TGT GGA CTT - 3′	0.7	
				p542S	6FAM - CAC CAA GTC CCT TAA AG - MGBNFQ - 3′	0.2	
Guanarito-TM	GP1	AY129247	73	F485	5′ - TGG ATT CTT GGG TGG ACA ATT AA - 3′	0.8	0.001
				R557	5′ - TAG GCT CAC AGC AGA TTC TTG GA - 3′	0.8	
				p532A1	6FAM - CCT CAA AAA GTC ATG TCC CAA TCC C - TAMRA - 3′	0.1	
Guanarito-TM	Polymerase	AY358024	80	F521	5′ - GCT GCC GGA GCT GTC TGA - 3′	0.7	0.01
				R599	5′ - ATG GTG CGA GTT TGT GGA CTT - 3′	0.7	
				p542S	6FAM - CAC CAA GTC CCT TAA AGA TTT GCT ATA GCA GAC AC - TAMRA -3′	0.1	
Hantavirus-MGB (Andes)	NP	AF291702	67	F41	5′ - GAA TGA GCA CCC TCC AAG AAT TG - 3′	0.9	10^{-5} (RNA dilution)§
				R107	5′ - CGA GCA GTC ACG AGC TGT TG - 3′	0.9	
				p71S	6FAM - ACA TCA CAG CAC ACG A - MGBNFQ	0.2	
Hantavirus-MGB (Sin Nombre and NY)	NP	L37904	71	F26	5′ - CTA CGA CTA AAG CTG GAA TGA GC - 3′	0.8	10^{-6} (SN)
				R96	5′ - GAG TTG TTG TTC GTG GAG AGT G - 3′	0.8	10^{-2} (NY)
				p59S	6FAM - AAG TGC AAG ACA ACA - MGBNFQ	0.2	(RNA dilution)§

* PCR = polymerase chain reaction; PFU = plaque-forming units; MGB = minor groove binder; NP = nucleoprotein; GP = glycoprotein; VP = virus protein; GPC = glycoprotein precursor.
† Limit of Detection (LOD) for all assays were measured in PFU/PCR unless otherwise stated; all sensitivities were done in triplicate and were positive in three of three replicates.
‡ Synthetic RNA was used to determine LODs in genome copy number (see Materials and Methods).
§ LODs measured as dilution of stock RNA because PFU information was not available.
¶ Machupo Mallele-TM assay run at 62°C.

One-Step RT-PCR Kit plus bovine serum albumin and ran on the LightCycler 2.0 (Roche Applied Science, Indianapolis, ID; www.roche.com). The amplicon size, primer/probe sequence, optimum assay concentration, and sensitivity (limit of detection [LOD]) are shown in Table 1. All assays were conducted at a final concentration of 3 mM $MgSO_4$ and the following cycling conditions: 50°C for 15 minutes (1 cycle); 95°C for 5 minute (1 cycle); 95°C for 1 second and 60°C for 20 seconds (45 cycles); and 40°C for 30 seconds (1 cycle). A single fluorescence read was taken at the end of each 60°C step. The Machupo Mallele TaqMan™ assay was conducted at 62°C for 45 amplification cycles.

Initial primer/probe down selection was accomplished by testing for cross-reactivity to individual members of the applicable virus family. Each pair that passed this test was then evaluated for optimum primer concentration by using an internal protocol. The final concentration selected resulted in the earliest C_t, lowest LOD, and highest end point fluorescence. Further exclusivity analysis involved a screening against two RNA panels: a United States Army Medical Research Institute of Infectious Diseases viral hemorrhagic fever panel containing infected cell lysate genomic RNAs of Ebola (Zaire, Sudan Gulu and Boniface, Reston, Ivory Coast, Bundibugyo), Marburg (Musoke, Angola, Ravn, Ci67), Lassa (Josiah, Weller, Macenta, Pinneo, Mobala, Mozambique), Machupo (Carvallo, Mallele), Junin (Romero), Sabia, and Guanarito viruses. The second arthropod-borne virus panel contained purified viral genomic RNAs from Venezuelan equine encephalitis viruses (VEE) IA/B (strain Trinidad donkey), VEE IC (CO951006), VEE ID (1D V209-A-TVP1163),VEE IE (68U201),VEE IF (78V3531), VEE II (Everglades Fe3-7c), VEE IIIA (Mucambo),VEE IV (BeAR40403),VEE V (Cabassou Be508), VEE VI (AG80-663), eastern equine encephalitis viruses (Georgia 97, ARG-LL and 76V-25343), western equine encephalitis virus (CBA 87/4), Barmah Forest (Aus BH2 2193), Nduma, Sindbis (UgMP6440), Highlands J, Mayaro (BEH256), Middleburg, Semliki Forest, yellow fever, Japanese encephalitis (B-0005/85), chikungunya (vaccine strain 15561), and Getah (Amm2021) viruses.

Sensitivity testing using cell culture supernatant viral genomic RNA with a known plaque-forming units (PFU) concentration determined the LOD of each assay. The LOD is defined as the lowest concentration of target RNA detected three of three times. The Lassa Macenta RNA PFU concentration was unknown. Therefore, the RNA was serially diluted 10-fold and the LOD was determined to be the lowest dilution detected three of three times. Additionally, for several assays, single-stranded full-length target RNA sequences synthesized by Dharmacon (Lafayette, CO; www.dharmacon.com), were used to determine the target copy number. A 10-fold serial dilution (1 pg–1 ag/PCR) was used to determine the LOD. The equivalent target copy number was calculated based on the molecular weight and length of the target sequence. Moreover, once the LOD was established, a standard curve could easily be used for an absolute quantitative real-time RT-PCR (qRT-PCR) analysis of unknown samples.

Although the viral hemorrhagic fever virus assays were designed using the Roche LightCycler 2.0, all assays should be easily transferable with minimal master mixture adjustments to most TaqMan™-compatible machines, including the Fast Real-Time PCR System (Applied Biosystems), the SmartCycler (Cepheid, Sunnyvale, CA; www.cepheid.com) the R.A.P.I.D and JBAIDS (Idaho Technology, Salt Lake City, UT; www.idahotech.com), the Mx QPCR (Stratagene, La Jolla, CA; www.stratagene.com) and the iCycler (Bio-Rad, Hercules, CA; www.bio-rad.com). Recent data indicated an easy transfer of both probe chemistries from one platform to another through similar assay performance, sensitivity, and specificity on three of these instruments.[20]

In a pilot evaluation of the TaqMan™-MGB assay designed to detect Lassa Josiah genomic RNA, 39 blood samples from patients infected with Lassa virus in Sierra Leone[21,22] were tested as part of the Lassa surveillance and treatment program based at Kenema Government Hospital.[23,24] Five of these samples were serially collected from one patient (#G-104) on days 1, 2, 3, 8, and 15 after admission to the Lassa Fever Ward. Viral genomic RNA was extracted from TRIzol-treated samples as described above and tested with the six Lassa TaqMan™-MGB assays (Josiah, Weller, Macenta, Pinneo, Mobala, and Mozambique). The resulting data from the LightCycler 2.0 software determined that blood of the patient was positive for Lassa Josiah on days 1, 2, 3, and 8 but was not detectable on day 15. An absolute quantification analysis of viral load indicates that 714, 633, 447 and 555 PFU/mL of patient blood were detected in samples on days 1, 2, 3 and 8, respectively. Of the remaining patient samples, 23 were positive for Lassa Josiah. Every Lassa Josiah–negative patient sample was also negative in the other five Lassa assays. All assays exhibited robust positive controls, and all negative controls were as expected. These results indicate that the six Lassa virus-specific TaqMan™-MGB assays were highly specific with the capability to detect potential clinically relevant levels of Lassa virus.

In conclusion, the assays presented in this report are highly specific and sensitive for detection of multiple hemorrhagic fever viruses. Rigorous design of primers and probes (length, melting point, amplicon size), stringent down selection, a strict optimization regiment, and common rapid-cycling parameters demonstrate that precise detection of multiple species/strains of filoviruses, arenaviruses, and New World hantaviruses (Sin Nombre and Andes viruses) is possible. Unknown samples can be tested simultaneously with multiple assays on TaqMan™-compatible instruments because these assays were designed, optimized, and validated by using identical cycling parameters. An additional benefit is the ability for researchers to multiplex the assays in any preferred combination.

Most importantly, because these are absolute qRT-PCR assays, researchers will be able to detect and identify unknown organisms in a sample and use the assays for viral load, vaccine, and antiviral drug studies. Additional validation of the assays with in-field samples will increase data on assay specificity and indicate their utility for clinical and environmental matrices. This collection of TaqMan™-based viral RNA real-time qRT-PCR assays provide a repertoire of diagnostic tools that can serve as a foundation for the simultaneous identification and analysis of these potential biothreat agents when multiple, rapid cycling, real-time PCR platforms are used.

Received October 21, 2009. Accepted for publication January 22, 2010.

Acknowledgments: We thank the technical staffs of Dr. Lisa E. Hensley and Dr. Randal J. Schoepp for supplying all viral genomic RNAs used in the experiments reported in this manuscript, Drs. Daniel Bausch and Robert F. Garry (Tulane University) for providing Lassa virus

RNA for assay evaluation, S. Coyne and P. Craw for work with TRIzol-extracted RNA with the Qiagen EZ1 robot, and Katheryn Kenyon for reviewing the manuscript.

Financial support: This study was supported by the Defense Threat Reduction Agency, project ID no. 8.10007_05_RD_B.

Disclaimer: The opinions, interpretations, conclusions, and recommendations in the report are those of the authors and are not necessarily endorsed by the U.S. Army.

Authors' addresses: Adrienne R. Trombley, Randal J. Schoepp, David A. Norwood, and David A. Kulesh, Diagnostic Systems Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD. Leslie Wachter, Medimmune, Gaithersburg, MD. Jeff Garrison, Battelle, Columbus, OH. Valerie A. Buckley-Beason and Lisa E. Hensley, Virology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD. Jordan Jahrling, Department of Neuroscience, University of Texas Medical Branch, Galveston, TX. Augustine Goba, Lassa Fever Laboratory, Kenema Government Hospital, Kenema, Sierra Leone. Joseph N. Fair, The Global Viral Forecasting Initiative, San Francisco, CA.

REFERENCES

1. Groseth A, Jones S, Artsob H, Feldmann H, 2005. Hemorrhagic fever viruses as biological weapons. Fong I, Alibek K, eds. *Bioterrorism and Infectious Agents: A New Dilemma for the 21st Century*. New York: Springer Publishing, 169–191.
2. Borio L, Inglesby T, Peters CJ, Schmaljohn AL, Hughes JM, Jahrling PB, Ksiazek T, Johnson KM, Meyerhoff A, O'Toole T, Ascher MS, Bartlett J, Breman JG, Eitzen EM, Jr., Hamburg M, Hauer J, Henderson DA, Johnson RT, Kwik G, Layton M, Lillibridge S, Nabel GJ, Osterholm MT, Perl TM, Russell P, Tonat K for the Working Group on Civilian Biodefense, 2002. Hemorrhagic fever viruses as biological weapons: medical and public health management. *JAMA 287:* 2391–2405.
3. Gibb TR, Norwood DA Jr, Woollen N, Henchal EA, 2001. Development and evaluation of a fluorogenic 5′ nuclease assay to detect and differentiate between Ebola virus subtypes Zaire and Sudan. *J Clin Microbiol 39:* 4125–4130.
4. Weidmann M, Muhlberger E, Hufert FT, 2004. Rapid detection protocol for filoviruses. *J Clin Virol 30:* 94–99.
5. Sanchez A, Lukwiya M, Bausch D, Mahanty S, Sanchez AJ, Wagoner KD, Rollin PE, 2004. Analysis of human peripheral blood samples from fatal and nonfatal cases of Ebola (Sudan) hemorrhagic fever: cellular responses, virus load, and nitric oxide levels. *J Virol 78:* 10370–10377.
6. Towner JS, Sealy TK, Khristova ML, Albarino CG, Conlan S, Reeder SA, Quan PL, Lipkin WI, Downing R, Tappero JW, Okware S, Lutwama J, Bakamutumaho B, Kayiwa J, Comer JA, Rollin PE, Ksiazek TG, Nichol ST, 2008. Newly discovered ebola virus associated with hemorrhagic fever outbreak in Uganda. *PLoS Pathog 4:* e1000212.
7. Towner JS, Rollin PE, Bausch DG, Sanchez A, Crary SM, Vincent M, Lee WF, Spiropoulou CF, Ksiazek TG, Lukwiya M, Kaducu F, Downing R, Nichol ST, 2004. Rapid diagnosis of Ebola hemorrhagic fever by reverse transcription-PCR in an outbreak setting and assessment of patient viral load as a predictor of outcome. *J Virol 78:* 4330–4341.
8. Drosten C, Gottig S, Schilling S, Asper M, Panning M, Schmitz H, Gunther S, 2002. Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean–Congo hemorrhagic fever virus, Rift valley fever virus, dengue virus, and yellow fever virus by real-time reverse transcription-PCR. *J Clin Microbiol 40:* 2323–2330.
9. Gibb TR, Norwood DA Jr, Woollen N, Henchal EA, 2001. Development and evaluation of a fluorogenic 5′-nuclease assay to identify Marburg virus. *Mol Cell Probes 15:* 259–266.
10. Trappier SG, Conaty AL, Farrar BB, Auperin DD, McCormick JB, Fisher-Hoch SP, 1993. Evaluation of the polymerase chain reaction for diagnosis of Lassa virus infection. *Am J Trop Med Hyg 49:* 214–221.
11. Demby AH, Chamberlain J, Brown DW, Clegg CS, 1994. Early diagnosis of Lassa fever by reverse transcription-PCR. *J Clin Microbiol 32:* 2898–2903.
12. Lunkenheimer K, Hufert FT, Schmitz H, 1990. Detection of Lassa virus RNA in specimens from patients with Lassa fever by using the polymerase chain reaction. *J Clin Microbiol 28:* 2689–2692.
13. Lozano ME, Enria D, Maiztegui JI, Grau O, Romanowski V, 1995. Rapid diagnosis of Argentine hemorrhagic fever by reverse transcriptase PCR-based assay. *J Clin Microbiol 33:* 1327–1332.
14. Garin D, Peyrefitte C, Crance JM, Le FA, Jouan A, Bouloy M, 2001. Highly sensitive Taqman PCR detection of Puumala hantavirus. *Microbes Infect 3:* 739–745.
15. Weidmann M, Rudaz V, Nunes MR, Vasconcelos PF, Hufert FT, 2003. Rapid detection of human pathogenic orthobunyaviruses. *J Clin Microbiol 41:* 3299–3305.
16. Botten J, Mirowsky K, Kusewitt D, Ye C, Gottlieb K, Prescott J, Hjelle B, 2003. Persistent Sin Nombre virus infection in the deer mouse (*Peromyscus maniculatus*) model: sites of replication and strand-specific expression. *J Virol 77:* 1540–1550.
17. Evander M, Eriksson I, Pettersson L, Juto P, Ahlm C, Olsson GE, Bucht G, Allard A, 2007. Puumala hantavirus viremia diagnosed by real-time reverse transcriptase PCR using samples from patients with hemorrhagic fever and renal syndrome. *J Clin Microbiol 45:* 2491–2497.
18. Aitichou M, Saleh SS, McElroy AK, Schmaljohn C, Ibrahim MS, 2005. Identification of Dobrava, Hantaan, Seoul, and Puumala viruses by one-step real-time RT-PCR. *J Virol Methods 124:* 21–26.
19. Coyne SR, Trombley A, Craw PD, Kulesh DA, Norwood DA, 2008. Extraction of RNA from virus samples in TRIzol using manual and automated magnetic bead systems. *American Society for Microbiology Biodefense and Emerging Diseases Research Meeting. Abstract 178 (B):* 60–61.
20. Christensen DR, Hartman LJ, Loveless BM, Frye MS, Shipley MA, Bridge DL, Richards MJ, Kaplan RS, Garrison J, Baldwin CD, Kulesh DA, Norwood DA, 2006. Detection of biological threat agents by real-time PCR: comparison of assay performance on the R.A.P.I.D., the LightCycler, and the Smart Cycler platforms. *Clin Chem 52:* 141–145.
21. McCormick JB, Webb PA, Krebs JW, Johnson KM, Smith ES, 1987. A prospective study of the epidemiology and ecology of Lassa fever. *J Infect Dis 155:* 437–444.
22. Khan SH, Goba A, Chu M, Roth C, Healing T, Marx A, Fair J, Guttieri MC, Ferro P, Imes T, Monagin C, Garry RF, Bausch DG, 2008. New opportunities for field research on the pathogenesis and treatment of Lassa fever. *Antiviral Res 78:* 103–115.
23. Bausch DG, Demby AH, Coulibaly M, Kanu J, Goba A, Bah A, Conde N, Wurtzel HL, Cavallaro KF, Lloyd E, Baldet FB, Cisse SD, Fofona D, Savane IK, Tolno RT, Mahy B, Wagoner KD, Ksiazek TG, Peters CJ, Rollin PE, 2001. Lassa fever in Guinea: I. Epidemiology of human disease and clinical observations. *Vector Borne Zoonotic Dis 1:* 269–281.
24. Bausch DG, Sesay SS, Oshin B, 2004. On the front lines of Lassa fever. *Emerg Infect Dis 10:* 1889–1890.