Improved Detection of Lassa Virus by Reverse Transcription-PCR Targeting the 5' Region of S RNA[∇]

Stephan Ölschläger,¹ Michaela Lelke,¹ Petra Emmerich,¹ Marcus Panning,^{1†} Christian Drosten,^{1‡} Meike Hass,¹ Danny Asogun,² Deborah Ehichioya,³ Sunday Omilabu,³ and Stephan Günther^{1*}

Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany¹; Irrua Specialist Teaching Hospital, Irrua, Edo State, Nigeria²; and Department of Medical Microbiology and Parasitology, College of Medicine, University of Lagos, Lagos, Nigeria³

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The method of choice for the detection of Lassa virus is reverse transcription (RT)-PCR. However, the high degree of genetic variability of the virus poses a problem with the design of RT-PCR assays that will reliably detect all strains. Recently, we encountered difficulties in detecting some strains from Liberia and Nigeria in a commonly used glycoprotein precursor (GPC) gene-specific RT-PCR assay (A. H. Demby, J. Chamberlain, D. W. Brown, and C. S. Clegg, *J. Clin. Microbiol.* 32:2898–2903, 1994), which prompted us to revise the protocol. The design of the new assay, the GPC RT-PCR/2007 assay, took into account 62 S RNA sequences from all countries where Lassa fever is endemic, including 40 sequences generated from the strains in our collection. The analytical sensitivity of the new assay was determined with 11 strains from Sierra Leone, Liberia, Ivory Coast, and Nigeria by probit analysis; the viral loads detectable with a probability of 95% ranged from 342 to 2,560 S RNA copies/ml serum, which corresponds to 4 to 30 S RNA copies/assay. The GPC RT-PCR/2007 assay was validated with 77 serum samples and 1 cerebrospinal fluid sample from patients with laboratory-confirmed Lassa fever. The samples mainly originated from Liberia and Nigeria and included strains difficult to detect in the assay of 1994. The GPC RT-PCR/2007 assay detected virus in all clinical specimens (100% sensitivity). In conclusion, a new RT-PCR assay, based in part on the protocol developed by Demby et al. in 1994, for the detection of Lassa virus is described. Compared to the assay developed in 1994, the GPC RT-PCR/2007 assay offers improved sensitivity for the detection of Liberian and Nigerian Lassa virus strains.

Lassa virus is a negative-strand RNA virus that belongs to the family *Arenaviridae*. It is endemic in West Africa and causes hemorrhagic fever in humans (8). An estimated 300,000 cases of Lassa fever occur annually (14). The natural host of Lassa virus is the small rodent *Mastomys natalensis*, which lives close to human settlements (11). Lassa virus may also be transmitted from human to human, which gives rise to nosocomial or community-based outbreaks. The virus has also been imported into countries where it is not endemic, for example, by returning travelers (1, 6, 7, 16). Laboratory testing is required to establish a diagnosis, as Lassa fever can hardly be distinguished from other febrile diseases on the basis of clinical symptoms (13). A suspect must be rapidly excluded or verified to facilitate appropriate case management, including treatment, the implementation of isolation measures, or the tracking of contact persons (9).

The method of choice for the early detection of Lassa virus in blood is reverse transcription (RT)-PCR (3, 12, 18). However, the high degree of genetic variability of the virus poses a problem with the design of RT-PCR assays for the reliable detection of all virus strains (2). In 1994, Demby et al. (3)

described an RT-PCR assay targeting the conserved terminus of the S RNA segment and the downstream glycoprotein precursor (GPC) gene. The primer design was based on several strains from Sierra Leone, Liberia, and Nigeria. The protocol was considered reliable and has been used by many laboratories for routine Lassa fever diagnostics (4).

However, in 2000 we noticed that a Lassa virus strain isolated from the cerebrospinal fluid (CSF) of a Nigerian patient showed seven exchanges in the reverse primer binding site, leading to a drop in the sensitivity of the GPC-gene RT-PCR (7). Similarly, in 2005 we experienced a diagnostic problem with serum samples from Liberian patients with Lassa fever from whom virus could be isolated in cell culture but who were false negative by the GPC-gene RT-PCR (M. Panning, unpublished data). Sequence analysis revealed mismatches at the 3' end of the reverse primer. Given the consequences of a false-negative Lassa virus test result for patient management and public health, these observations prompted us to revise the GPC-gene RT-PCR protocol. The analytical and clinical performance characteristics of the new assay have been thoroughly validated.

MATERIALS AND METHODS

Clinical samples. A total of 77 serum samples and 1 CSF sample from patients with laboratory-confirmed Lassa fever were available. Sixty-eight samples were from Liberian patients, six were from Nigerian patients, and four were from imported cases of Lassa fever (Ivory Coast, 2000 [6]; Nigeria, 2000 [7]; Sierra Leone, 2000 and 2006 [1, 16]). All samples were positive by virus culture and/or published RT-PCR assays (4, 19) and, except for the six samples from Nigerian patients, had been processed through the routine diagnostic service of the Bernhard Nocht Institute between 2000 and 2008.

* Corresponding author. Mailing address: Bernhard Nocht Institute for Tropical Medicine, Department of Virology, Bernhard-Nocht-Strasse 74, Hamburg 20359, Germany. Phone: 49 4042818 421. Fax: 49 4042818 378. E-mail: guenther@bni.uni-hamburg.de.

† Present address: Abteilung für Virologie, Universitätsklinikum Freiburg, Freiburg, Germany.

‡ Present address: Institute of Virology, University of Bonn Medical Centre, Bonn, Germany.

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Virus culture. Virus was propagated in the biosafety level (BSL) 4 laboratory of the Bernhard Nocht Institute. For the isolation of virus from clinical specimens, Vero cells were seeded in 25-cm² tissue culture flasks in Dulbecco's minimum essential medium and inoculated with an aliquot of serum. Virus growth was monitored by immunofluorescence with a Lassa virus nucleoprotein (NP)-specific monoclonal antibody.

RNA extraction. Viral RNA was extracted from serum, CSF, or cell culture supernatant with a QIAamp viral RNA minikit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA was eluted in 60 µl AVE buffer (Qiagen) and stored at -70°C. The nucleic acid of HeLa cells was extracted with RNeasy columns (Qiagen), according to the manufacturer's instructions, quantified spectrophotometrically, and stored at -70°C.

Partial sequencing of S RNA. The 5' portion of S RNA (positions 20 to 1000), which encompasses the target region of the GPC RT-PCR, was amplified with pan-Old World arenavirus primers and a Qiagen OneStep RT-PCR kit (Qiagen). The 20-µl assay mixture contained 2 µl RNA, 0.5 µM primer OWS-1-fwd (GCG CAC CGG GGA TCC TAG GC), 0.5 µM primer OWS-1000-rev (AGC ATG TCA CAA AAY TCY TCA TCA TG), 0.4 mM deoxynucleoside triphosphate (dNTP), 1× RT-PCR buffer, and 0.8 µl enzyme mixture. The reaction was performed in a Primus25 advanced thermocycler (PiqLab, Erlangen, Germany) using the following temperature profile: 50°C for 30 min and 95°C for 15 min, followed by 45 cycles of 95°C for 20 s, 55°C for 20 s, and 72°C for 1 min. Both strands of the amplified fragments were sequenced.

Identification of conserved primer binding sites. Lassa virus sequences available from GenBank database and those generated in this study were aligned with the BioEdit (version 7.0.5) program (10). Conserved sites in the 5' region of the GPC gene suitable for primer binding were identified by visual inspection.

Cloning of GPC RT-PCR target region and generation of *in vitro* transcripts. The 1-kb amplicons of the pan-Old World arenavirus PCR were cloned into a T7 polymerase expression vector. Alternatively, the target region (positions 1 to 400 of S RNA) of viruses not available in our strain collection (Lassa virus strains GA391, LP, 803213, and Weller) was chemically synthesized and cloned by the Genart Company, Regensburg, Germany. The correct sequence of the inserts was ascertained by sequencing. The complete insert, including the T7 promoter upstream of the insert, was amplified from a small amount of plasmid using plasmid-specific primers. The target region was transcribed from the PCR product *in vitro* using a MEGAscript high yield transcription kit (Ambion, Austin, TX), according to the manufacturer's instructions. The RNA was purified with RNeasy columns (Qiagen), quantified spectrophotometrically, and stored at -70°C.

Diagnostic Lassa virus GPC RT-PCRs. The new GPC RT-PCR (called GPC RT-PCR/2007) employed OneStep RT-PCR kit reagents (Qiagen). The 25-µl assay mixture contained 5 µl RNA, 0.6 µM primer 36E2 (ACC GGG GAT CCT AGG CAT TT), 0.6 µM primer LVS-339-rev (GTT CTT TGT GCA GGA MAG GGG CAT KGT CAT), 0.4 mM dNTP, 1× RT-PCR buffer, 1× Q solution, and 1 µl enzyme mixture. The cycling conditions were 50°C for 30 min and 95°C for 15 min, followed by 45 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s. The assay was set up on ice and placed into a Primus25 advanced thermocycler (PiqLab), after the heating block had reached 50°C.

The protocol published in 1994 by Demby et al. (called GPC RT-PCR/1994) (3) was used in conjunction with the same enzymatic platform and reaction conditions described above for the GPC RT-PCR/2007 assay. In the diagnostic setting, the GPC RT-PCR/1994 assay was performed according to the protocol published by Drosten et al. in 2002 (4).

Statistical analysis. The 95% detection limit of the RT-PCR was determined by probit analysis by using the Probit (version 1.63) program (15). The experimental input data for this nonlinear regression model were the different test concentrations of RNA and the corresponding proportion of positive results after replicate PCR testing.

Nucleotide sequence accession numbers. The sequences reported in this paper have been sent to the GenBank database and assigned accession nos. GU481063, GU481068, GU481070, GU481072, GU481074, GU481076, GU481078, GU830812 to GU830843, and GU979508.

RESULTS

Virus sequencing. The 5' portion of S RNA encompassing the target region of the GPC RT-PCR/1994 assay was sequenced for one strain from Sierra Leone (strain SL06-2057 [GenBank accession no. GU979508]) (1), 6 strains from Guinea (strains BA289, BA366, BA377, BA384, DGD43, and TA491 [GenBank acces-

sion nos. GU830838 to GU830843, respectively]) (11), 26 strains from Liberia (strains Lib04-2739, Lib05-236/88, Lib05-406/90, Lib05-1580/121, Lib05-2096/127, Lib05-2406/129, Lib05-3800, Lib05-4094, Lib06-120, Lib06-174, Lib06-295, Lib06-383, Lib06-1099, Lib06-1101, Lib06-1826, Lib06-2442, Lib07-29, Lib07-30, Lib07-54, Lib07-55, Lib07-56, Lib07-206, Lib07-444, Lib07-515, Lib07-612, and Lib07-624 [GenBank accession nos. GU830812 to GU830837, respectively]), and 7 strains from Nigeria (strains Nig08-A18, Nig08-A19, Nig08-A37, Nig08-A41, Nig08-A47, Nig08-02, and Nig08-04 [GenBank accession nos. GU481063, GU481068, GU481070, GU481072, GU481074, GU481076, and GU481078, respectively]). Newly generated sequences as well as sequences from GenBank available by the end of 2007 were considered in the primer design. Strains and sequences which became available at a later time were used to verify the primer design (Fig. 1) and were included in the assay validation (Table 1).

Primer design. Forward primer 36E2 of the original GPC RT-PCR/1994 assay was kept, as there was no evidence for mismatches with this primer. Reverse primer 80F2, which showed several mismatches with Liberian and Nigerian Lassa virus strains and which was responsible for the problems with our diagnostics, was replaced by primer LVS-339-rev. The amplicon size of the new assay was 318 nucleotides for Lassa virus Josiah (positions 4 to 322 of S RNA). A wobble base (K = G and T) was introduced at position -6 of the primer to compensate for the variability at the 3' end of the primer. Thus, the sequences of most virus strains perfectly matched the first 11 positions of the primer (Fig. 1). A second wobble was introduced at primer position -15 (M = A and C). The protocol used for the new assay, designated the GPC RT-PCR/2007 assay, is described in Materials and Methods.

Sensitivity of GPC RT-PCR/2007 compared to that of GPC RT-PCR/1994. To verify that the new protocol detects potentially difficult templates, it was tested with a panel of 19 isolates mainly originating from Guinea, Liberia, and Nigeria. The same panel was tested by the GPC RT-PCR/1994 assay. RNA was prepared from the supernatants of virus cultures, diluted in 10-fold steps, and amplified in parallel by both protocols. All strains were detected by the new protocol at dilutions ranging from 10⁻² to 10⁻⁷. The GPC RT-PCR/2007 assay performed as well as or better than the GPC RT-PCR/1994 assay. Improved sensitivity was observed for strains from Liberia and Nigeria (Table 1).

Analytical sensitivity of GPC RT-PCR/2007. For the statistically precise determination of the detection limit in terms of RNA copy numbers, the target regions of a representative set of Lassa virus strains covering all known genetic lineages were cloned: strain NL from Sierra Leone; strains Lib05-406/90, Lib05-1580/121, Lib06-120, and Lib06-295 from Liberia; strain AV from Ivory Coast; and strains CSF, 803213, LP, Weller, and GA391 from Nigeria (Table 2). The transcripts were synthesized and quantified *in vitro*. Different amounts of the RNA transcripts were spiked into human serum prior to RNA preparation, and each RNA sample was tested in 5 to 15 replicates. For each strain, the test concentrations of RNA and the corresponding proportion of positive results after replicate testing were subjected to probit regression analysis. The statistics revealed that the GPC RT-PCR/2007 assay detects between 342 and 2,560 RNA copies/ml serum, depending on the virus

Lassa virus strains	321	292
	5' -GTTCTTTGTGCAGGAMAGGGGCATKGTTCAT-3'	
Sierra Leone (4)		
Josiah	G..A.....	VC
331, NL, SL06-2057	A..G..A.....	P,S
Guinea (6)		
BA377	A..T.....A..G.....A.....	VC
BA366, BA289, BA384	...T.....G..A.....	VC
TA491	...T.....A.....G..A.....	VC
DGD43	A.....	VC
Liberia (29)		
Z148, Macenta	A..G.....	
Lib06-174	A.....A..G.....	S
Lib07-515	A..G..A.....	S
Lib05-236/88, Lib05-406/90	A..A..G..A.....	P,S
523, Lib06-1826	G..A.....	S
Lib05-4094	...T.....G..A.....	S
Lib07-624	...T...A.....G..A.....	S
Lib05-1580/121	...T...A.....A.....	P,VC,S
Lib05-2406/129	...T...A.....A.....	VC,S
Lib06-120	...T...A.....A.....	P,S
Lib05-2096/127, Lib07-54, Lib07-206, Lib07-612	...T.....A.....A.....	VC,S
Lib06-295, Lib06-1099	A..A.....A.....	P,S
Lib06-383, Lib07-30, Lib07-444	A.....A.....	VC,S
Lib06-2442	C.....A.....	S
Lib07-29, Lib07-55, Lib07-56	A.....	S
Lib06-1101	A.....A.....	S
Lib04-2739	A.....	
Lib05-3800	A..A.....	S
Mali (1)		
Soromba	...T..G.....A..G.....A.....	
Ivory Coast (1)		
AV	G.....	P,VC,S
Nigeria (21)		
Nig08-A41	A..T..A.....	VC,S
Nig08-A47	A.....A..T..A.....	VC,S
PA, Nig04-10	...T.....A..T..A.....	
Nig08-A37	C.....T..A.....	VC,S
Pinneo, LP	G.....T..T..A.....	P
GA391, Nig08-02	T..C.....	P,S
Nig08-A18	A.....T..C.....	VC,S
Nig08-A19	A..A..T..C.....A.....	VC,S
Nig08-04	...T...A..A..T..A.....A.....	VC,S
PB1	...T.....T..A.....	
Nig09-OSPMH-123	...T.....A..T..T.....	S
Nig09-OSPMH-86	..CT...C...A.....T.....	S
Nig09-OSPMH-89, Nig09-OSPMH-124	...T.....T.....	S
CSF	A..T.....A.....A.....	P,VC,CSF
SA, Weller	AT.....A.....	P
803213	C..A..A.....A.....C.....	P

FIG. 1. Binding site of primer LVS-339-rev in different Lassa virus strains. The primer sequence is indicated at the top of the alignment in the 5' to 3' orientation. The positions are numbered according to the S RNA of Lassa virus strain Josiah. The primer contains two wobble bases (underlined), and dots at these positions in the alignment represent identities to either of the two possible bases (M = A or C, K = G or T). Experimental evidence for the ability of the GPC RT-PCR/2007 assay to detect one or several of the strains is summarized on the right: probit (P), the *in vitro* transcript of S RNA was used to determine the 95% detection limit (Table 2); virus culture (VC), the isolate was detected in the supernatant of the virus culture (Table 1); serum (S) and CSF, virus was detected in serum or CSF from infected humans or animals. The strain Nig09-OSPMH sequences were kindly provided by S. Omilabu (unpublished data).

strain, with a probability of 95% (Table 2). This 95% detection limit corresponds to 4 to 30 S RNA copies per reaction, considering that RNA was prepared from 140 μ l serum and 1/12th of the RNA preparation was used as the template and assuming a 100% efficiency of RNA preparation. The pooled data for all strains tested (326 data points) resulted in an overall 95% detection limit of 1,237 Lassa virus RNA copies/ml (95% confidence interval, 974 to 1,892 copies/ml) (Fig. 2).

Analytical specificity. The nucleic acids of other hemorrhagic fever viruses, including Crimean Congo hemorrhagic fever virus, Ebola virus, Marburg virus, Rift Valley fever virus, dengue virus types 1 to 4, and yellow fever virus, were extracted from cell culture material or clinical specimens and tested by

the GPC RT-PCR/2007 assay. In addition, other viruses or parasites which may circulate in the blood of patients in the tropics, such as HIV, hepatitis C virus, hepatitis B virus, Chikungunya virus, Epstein-Barr virus, herpes simplex virus type 1, and *Plasmodium falciparum*, were tested. None of the specimens tested positive or yielded signals that could interfere with interpretation of the assay (data not shown). To investigate the effects of large amounts of human DNA and RNA on the performance of the assay, nucleic acid was extracted from HeLa cells. The extract (2.2 μ g, 1.5 μ g, and 0.7 μ g nucleic acid per reaction mixture) was mixed with 100 copies of *in vitro*-transcribed Lassa virus RNA and tested by the GPC RT-PCR/2007 assay. Addition of up to 2.2 μ g of HeLa cell nucleic acid

TABLE 1. Sensitivity of GPC RT-PCR/2007 compared to that of GPC RT-PCR/1994^a

Lassa virus strain	Origin of strain	End-point titer difference between GPC RT-PCR/2007 and GPC RT-PCR/1994
Josiah	Sierra Leone	+1
BA289	Guinea	+1
BA366	Guinea	0
BA377	Guinea	+1
BA384	Guinea	0
TA491	Guinea	+1
DGD43	Guinea	0
Lib05-1580/121	Liberia	+2
Lib05-2096/127	Liberia	0
Lib05-2406/129	Liberia	+2
Lib07-444	Liberia	0
AV	Ivory Coast	0
Nig08-A18	Nigeria	+2
Nig08-A19	Nigeria	+1
Nig08-A37	Nigeria	0
Nig08-A41	Nigeria	0
Nig08-A47	Nigeria	+1
Nig08-04	Nigeria	0
CSF	Nigeria	≥ +5

^a Virus RNA was tested in serial 10-fold dilutions. The highest dilution step yielding a signal was defined as the end-point titer. The table shows the end-point titer obtained by GPC RT-PCR/2007 minus the end-point titer obtained by GPC RT-PCR/1994 (i.e., positive values indicate that the former is more sensitive than the latter). Due to the statistical distribution of positive results at the limit of detection, only titer differences of ≥ 2 (RNA concentration difference ≥ 100 -fold) should be considered significant (shown in boldface).

did not affect either the amplification of the Lassa virus RNA sequences (100 copies) or interpretation of the assay (data not shown).

Validation with clinical gold standard. The GPC RT-PCR/2007 assay was validated with 77 serum samples and 1 CSF sample from patients with laboratory-confirmed Lassa fever. Laboratory confirmation was defined as virus detection by published PCR assays (4, 19) and/or virus isolation in cell culture (the “gold standard” assays). The collection included four virus culture-positive serum samples from Liberian patients that were false negative by the GPC RT-PCR/1994 assay (4). The GPC RT-PCR/2007 assay detected virus in all 78 samples (see

TABLE 2. Detection limit of GPC RT-PCR/2007 for different Lassa virus strains determined by replicate testing and probit analysis

Lassa virus strain	Origin of strain	Genetic lineage	95% detection limit	
			No. of RNA copies/ml sample	No. of RNA copies/reaction mixture
NL	Sierra Leone	IV	536	6.3
Lib05-406/90	Liberia	IV	2,560	29.9
Lib05-1580/121	Liberia	IV	342	4.0
Lib06-120	Liberia	IV	686	8.0
Lib06-295	Liberia	IV	975	11.4
AV	Ivory Coast	V	820	9.6
CSF	Nigeria	III	870	10.2
Weller	Nigeria	III	2,067	24.1
GA391	Nigeria	III	1,080	12.6
803213	Nigeria	II	782	9.1
LP	Nigeria	I	1,372	16.0

FIG. 2. Analytical sensitivity of the GPC RT-PCR/2007 assay determined by probit analysis. The analysis was performed with pooled data, shown in Table 2, for all 11 virus strains. Filled squares, experimentally determined fractions of positive results (y axis) at a given RNA concentration (x axis); solid line, regression curve for the probability of obtaining a positive RT-PCR result at a given concentration of Lassa virus RNA in plasma; right and left dotted lines of the regression curve, the 95% confidence interval; cop, number of copies.

Fig. 3 for relevant examples). Thus, by comparison with the results of our gold standard assays, the clinical sensitivity of the GPC RT-PCR/2007 assay was 100%.

DISCUSSION

Here we describe a new RT-PCR assay for the detection of Lassa virus. The assay is based in part on the protocol developed in 1994 by Demby et al. (3). Compared to the latter assay, the new GPC RT-PCR/2007 assay shows improved sensitivity for the detection of some Liberian and Nigerian Lassa virus strains.

Several PCR assays for the specific detection of Lassa virus have been published in the past (3, 12, 17, 18). However, these

FIG. 3. Detection of Lassa virus in Liberian serum samples by using the GPC RT-PCR/2007 assay. RNA was extracted from serum and tested undiluted (ud) and at a 1:10 dilution. The results obtained by the GPC RT-PCR/1994 assay according to the protocol published in 2002 (4) for routine diagnosis are shown below the agarose gel. Samples Lib05-1580/121 and Lib05-2406/129 were positive by virus culture. NTC, nontemplate control; Pos. Control, positive control. The numbers on the right are in base pairs.

assays had to be established on the basis of the few sequences available in the 1990s, and it was soon noted that some Lassa virus strains escape detection by PCR (18). In 2003, an *in silico* evaluation showed that some PCR primers published for diagnostic use do not detect all Lassa virus strains (5). The protocol developed in 1994 by Demby et al. (3) has been considered reliable, until we noticed problems with the detection of Nigerian and Liberian strains.

The design of the new GPC RT-PCR/2007 assay took into account 62 S RNA sequences from all countries where Lassa fever is endemic. For the vast majority of these sequences, we provide experimental evidence that the corresponding virus RNA is detected by the GPC RT-PCR/2007 assay (Fig. 1, right). In addition, the binding site of the new reverse primer overlaps with a conserved sequence characterized by a low level of redundancy in codon usage. The amino acid sequence underlying the binding site of the primer's 3' end is Met-Thr-Met-Pro, which is conserved among all known Lassa virus strains. Reverse translation of the sequence reveals ATG-ACN⁻⁶-ATG-CCN⁻¹². Thus, as long as the amino acid sequence is conserved, there is only 1 variable nucleotide position (position -6) among the first 11 positions of the primer LVS-339-rev binding site (this is mainly because Met is coded for by a single codon). Variability at the -6 position was taken into account to some extent by introducing a wobble base in the primer. While these features should render the assay robust in terms of virus variability, there is no guarantee that it will detect unknown Lassa virus strains.

The analytical sensitivity of the GPC RT-PCR/2007 assay is high and nearly in the range of sensitivities of commercial assays. The 95% detection limit somehow differs for various strains (342 to 2,560 RNA copies/ml serum), which may be explained by the statistical imprecision of the estimate due to the low number of replicates per dose level. The detection limit of 1,237 copies/ml serum (15 copies per reaction) calculated with 326 data points from all experiments provides a robust estimate of the overall sensitivity. Most importantly, the high degree of analytical sensitivity for the detection of all strains, including the difficult-to-detect Liberian and Nigerian strains, resulted in 100% clinical sensitivity when the assay was tested by use of our gold standard collection of clinical samples. Due to the convincing validation data, the GPC RT-PCR/2007 assay has been implemented in the routine diagnostics of the Bernhard Nocht Institute in Germany, the Virology Research Unit at the University of Lagos, and the Institute for Lassa Fever Research and Control at the Irrua Specialist Teaching Hospital in Nigeria.

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