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VIROLOGY

Ebola Preparedness: Diagnosis Improvement Using Rapid Approaches for Proficiency Testing

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ABSTRACT The unprecedented 2015 Ebolavirus (EBOV) outbreak in West Africa was declared a public health emergency, making diagnosis and quality of testing a global issue. The accuracy of laboratory diagnostic capacity for EBOV was assessed in 2014 to 2016 using a proficiency testing (PT) strategy developed by the Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP) in Biosecurity. Following a literature search, EBOV-specific gene targets were ranked according to the frequency of their use in published methods. The most commonly used gene regions (nucleoprotein [NP], glycoprotein [GP], and RNA-dependent RNA polymerase [L]) were selected for the design of *in vitro* RNA transcripts to be included in the simulated EBOV specimens used for EBOV detection with PCR-based assays. Specimens were tested for stability and found to be stable on long-term storage (1 year) at −80°C and on shorter-term storage in lyophilized form (1 week at ambient temperature and a subsequent week at −80°C). These specimens were used in three EBOV PTs offered from April 2014 to March 2016. In the first and third PTs, all laboratories (3/3 and 9/9, respectively) correctly identified specimens containing EBOV RNA transcripts, while in the second PT, all but one laboratory (5/6) correctly confirmed the presence of EBOV. The EBOV PT panel was useful for ensuring the competency of laboratories in detecting EBOV in the absence of readily available clinical samples. The simulated EBOV specimen was safe, stable, and reliable and can be used in lyophilized form for future EBOV PT programs, allowing simplicity of transport.

KEYWORDS Ebola diagnosis, Ebolavirus, proficiency testing

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The first identification of Ebolavirus disease (EVD) was in 1976 during two separate large outbreaks in the Democratic Republic of Congo (formerly known as Zaire) and Sudan. The initial outbreak resulted in the discovery of two distinct species of Ebolavirus, *Zaire ebolavirus* (EBOV) and *Sudan ebolavirus* (SUDV), which have since been the major species responsible for most subsequent EVD outbreaks. Of the remaining three species of Ebolavirus, *Taï Forest ebolavirus* (TAFV) and *Bundibugyo ebolavirus* (BDBV) less frequently cause outbreaks, and *Reston ebolavirus* (RESTV) is confined to nonhuman primate infections. The most recent, and also the largest, outbreak was the first in West Africa and was due to the Zaire species (1). This outbreak began in December 2013 and was first detected in February 2014 in Guinea (2), with the last cases reported between 1 and 5 April 2016 from Monrovia in Liberia. While Guinea declared an end to Ebolavirus transmission on 1 June 2016, the World Health Organization (WHO) declared the end of the most recent outbreak of EVD in Liberia on 9 June 2016.

Ebolaviruses are select agents and WHO risk group 4 pathogens. They are categorized as category A bioterrorism agents by the U.S. Centers for Disease Control and

Prevention (CDC) and are listed as tier 1 security sensitive biological agents (SSBA) under the Australian National Health Security Act of 2007. As they cause rapid and severe diseases accompanied by high mortality rates, they require testing to be performed in physical containment level 4 (PC4) or biosafety level 4 (BSL4) facilities. The Ebolavirus genome is 19 kb in length, encoding seven known structural proteins: nucleoprotein (NP), polymerase cofactor (VP35), matrix protein (VP40), glycoprotein (GP), replication-transcription protein (VP30), minor matrix protein (VP24), and RNA-dependent RNA polymerase (L). Highly specific, sensitive, and reliable procedures for the diagnosis of filoviral hemorrhagic fevers are based predominantly on multiple genome targets, using real-time reverse transcription-PCR (real-time RT-PCR) assays. Real-time RT-PCR assays are able to detect viral RNA in blood specimens (3–8) and to estimate viral loads in blood, which correlate with clinical outcome (9–12). They are the most suitable diagnostic tests at the early stages of disease due to the presence of high titers of virus in blood. False-negative or false-positive results are critical, as such errors will cause delays in the initiation of appropriate public health measures, and hence accurate testing is critical for personal and community protection. Further, regions where outbreaks of EVD occur are also areas of endemicity of other PC4- and BSL4-level pathogens with high public health risk, such as *Lassa virus*, *Crimean-Congo hemorrhagic fever virus*, *Dengue virus*, *Yellow fever virus*, and *Plasmodium falciparum*, which may cause similar clinical diseases, particularly during the initial stages of infection (13).

Although the public health emergency of international concern (PHEIC) related to Ebola in West Africa was lifted on 29 March 2016, there remains a need for ensuring EVD preparedness due to the threat of imported cases. Given the fact that a single misidentification of EVD has enormous political, economic, and human consequences, the quality of testing for EBOV has become a major global issue. External quality assurance programs are an essential tool for monitoring the diagnostic proficiency of laboratories and providing results that allow implementation of improved testing, thereby strengthening global capability for reducing spread. Appropriate proficiency testing (PT) programs are one major approach to reducing the risk of misdiagnosis by the laboratory. They require a high level of confidence that results of nucleic acid testing (NAT) for EBOV-specific genes in diagnostic settings are not compromised by the quality or stability of the PT specimens.

Here, the Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP) in Biosecurity developed a PT program for the detection of EBOV. The absence of readily available and inactivated, low-risk clinical samples has resulted in the need for simulated EBOV specimens consisting of RNA transcripts. These transcripts were generated using *in vitro* T7 transcription of plasmids containing two fragments of the EBOV GP gene and one fragment each of the EBOV NP and L genes. The purpose of the PT program was to evaluate the performance of the laboratories in detecting the presence of EBOV using PCR-based NAT. Over a period of 2 years, three rounds of this PT program were offered to 10 participating laboratories and generally demonstrated accuracy in detecting EBOV using NAT. This program offers PT in the absence of inactivated EBOV, using simulated EBOV specimens that are safe to handle and stable after storage. This is also the first time that such a program has been documented. It highlights the importance of an effective EBOV diagnosis, accompanied by the use of well-characterized, noninfectious simulated specimens, in designing a reliable PT to assess laboratory competency to detect, analyze, and report the presence of EBOV.

RESULTS

Stability of the simulated EBOV specimen. No significant degradation was observed in the simulated specimen containing the EBOV-specific *in vitro* RNA transcripts after 1 year of storage at −80°C (Fig. 1A). There was no significant decrease in the relative number of genome equivalent (GE) copies/ml for each *in vitro* RNA transcript (EBOV NP, $P = 0.3175$; EBOV GP1, $P = 0.0952$; EBOV GP2, $P = 0.5238$; EBOV L, $P = 0.2222$) in the simulated EBOV specimen. Similarly, the lyophilized simulated EBOV specimen was not significantly degraded over the course of a 2-week short-term

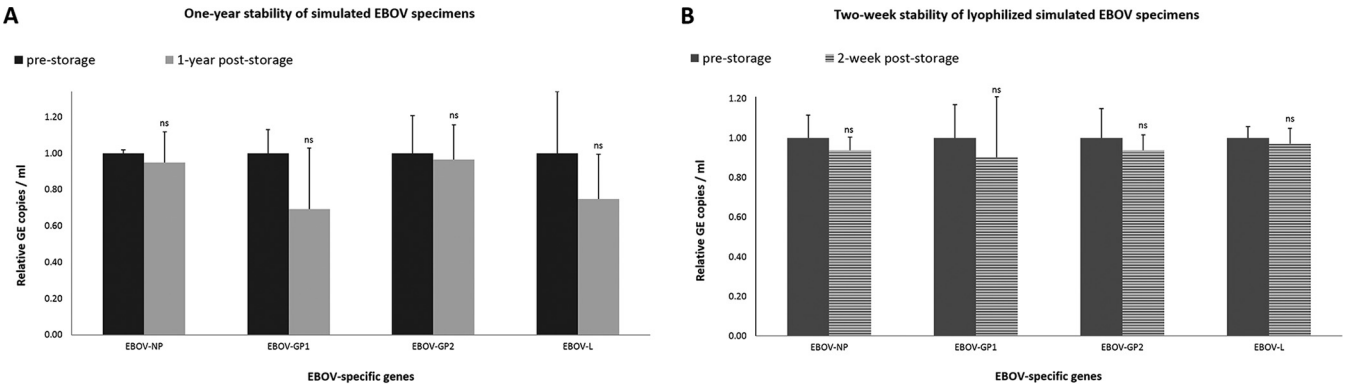

FIG 1 Stability of the simulated EBOV specimens based on the comparison of relative genome equivalents (GE) copies/ml pre- and poststorage of EBOV NP, EBOV GP1, EBOV GP2, and EBOV L (calculated by dividing the number of GE copies/ml prestorage by the number of GE copies/ml poststorage) and testing at 1 year following storage at −80°C in a 500-μl mixture (A) and following 1 week of storage at ambient temperature and a subsequent 1 week at −80°C in lyophilized form (B). The variation of data (mean ± SD) was from five randomly selected replicates ($n = 5$) (one-year stability), and three randomly selected replicates ($n = 3$) (two-week stability) of the simulated EBOV specimen, prepared at an average of approximately 1.0×10^8 GE copies/ml. The two-tailed Mann-Whitney U test was used to determine significance ($P < 0.05$). ns, not significant.

storage, i.e., a week at ambient temperature followed by another week at −80°C (Fig. 1B). These lyophilized specimens with short-term (2-week) storage appeared to be stable, with no significant decrease in the relative number of GE copies/ml of each *in vitro* RNA transcript in the lyophilized specimen (EBOV NP, $P = 0.7$; EBOV GP1, $P = 0.9$; EBOV GP2, $P = 0.7$; EBOV L, $P = 0.7$) compared to the relative number of GE copies/ml prestorage.

Performance of the participating laboratories in the PT program. Over the course of 2 years (April 2014 to March 2016), the EBOV proficiency testing (PT) program was offered to a total of 10 participating laboratories. Three laboratories in Australia participated in the EBOV PT when it was first offered in April 2014. The participation increased in the second PT program within the same year, which was offered in November 2014, with a total of six laboratories. The most recent EBOV PT was offered in March 2016, and the participation included seven Australian laboratories and two international laboratories (Table 1).

Performance for the detection and identification of EBOV was assessed qualitatively and quantitatively. The summary of the results for both specimens included in the PT panels and the overall performance of participating laboratories are presented in Table 1. The gene targets used by laboratories to confirm or rule out the presence of EBOV in both of the PT specimens were NP, GP, and L. In the first (April 2014) and third (March 2016) EBOV PTs, all laboratories correctly reported the presence of EBOV in item 1, and no false-positive results were reported for NAT performed on item 2, which did not contain any genetic material. In the second PT program (November 2014), all laboratories but one correctly reported the presence of EBOV in item 1. The only laboratory (participant 3) that submitted a wrong result had ruled out EBOV in item 1 (false negative) but confirmed EBOV to be present in item 2 (false positive).

Methods used for the detection of EBOV. Protocols to confirm or rule out the presence of EBOV differed between laboratories. However, examination of the set of EBOV gene targets that were tested in the PT program showed most laboratories used an in-house assay, except for one laboratory (participant 3) which also used a commercial kit, the RealStar Ebolavirus RT-PCR kit 1.0 (Altona Diagnostics GmbH), in the second EBOV PT. Another laboratory (participant 1) used a kit registered by the U.S. Food and Drug Authorization (FDA) through the issue of the Emergency Use Authorization, as supplied by the U.S. CDC, in the third PT program (the gene target was undisclosed). All protocols used by participating laboratories detected a minimum of one and a maximum of three EBOV-specific gene targets (Table 1). Real-time PCR assays were the most commonly used PCR method, while some laboratories included conventional gel-based PCR assays in their testing regimen (data not shown).

TABLE 1 Summary of results for simulated EBOV specimen (item 1) included in three PTs and overall performance of participating laboratories

Date	Participant	EBOV[a]	EBOV-specific targets[b]		
			NP	GP	L
April 2014	1	Y	D	—	D
	2				
	3				
	4	Y	D	—	D
	5				
	6				
	7				
	8				
	9				
	10	Y	—	D	—
November 2014	1	Y	D	—	D
	2	Y	D[c]	D	—
	3	N[d]	—	—	ND
	4	Y	D[c]	D	D[c]
	5				
	6				
	7	Y	D	D	D
	8				
	9				
	10	Y	D	D	D
March 2016	1	Y[e]			
	2	Y	D	D	—
	3	Y	—	—	D
	4	Y	D[c]	D	D[c]
	5	Y	D	D	—
	6	Y	D	—	—
	7				
	8	Y	D	D	D
	9	Y	D[c]	D[c]	—
	10	Y	D	D	D

[a]Y, present; N, not present.
[b]D, target detected; ND, target not detected; —, target not tested.
[c]A result from a second EBOV-specific NAT assay was reported.
[d]The laboratory reported the use of RealStar Ebolavirus RT-PCR kit 1.0 (Altona Diagnostics GmbH; authorized by the U.S. FDA through the issue of the Emergency Use Authorization). Item 2 in the same proficiency testing, which did not contain any genetic material, was reported as having EBOV-specific targets detected.
[e]The laboratory reported the use of a kit (as supplied by the U.S. CDC; the gene target was undisclosed).

DISCUSSION

This report presents data on the utility of the rapidly instituted PT program in monitoring the proficiency of laboratories in diagnosing EBOV specimens and in allowing the implementation of improved molecular testing in laboratories. Accurate diagnosis, predicated upon regular proficiency testing, reduces the risk from EBOV infection at the community and public health levels. Here, three EBOV PTs were offered from April 2014 to March 2016 during the EBOV outbreak in West Africa. The first EBOV PT, offered in April 2014, included three laboratories, which increased to six laboratories in November 2014 and finally to nine laboratories worldwide in March 2016. As the simulated EBOV specimens were made available, additional laboratories subsequently acquired the ability to develop real-time RT-PCR assays in-house. These laboratories consequently participated in the second and third EBOV PTs. Among all 10 participating laboratories, only one reported incorrect results (false positive and false negative), which was attributed to incorrect interchanging of specimens, as the laboratory reported the presence of EBOV in a negative specimen and the absence of EBOV in the simulated EBOV specimen. This highlights the importance of PT to determine the competency of participating laboratories not only in correctly testing and identifying the specific agent but also in specimen handling capability.

While accurate EBOV diagnosis can be monitored and ensured through a regular assessment of laboratory capability in a PT program, a high level of confidence in the

quality or stability of the PT specimens included in the PT panel remains fundamental to confirm the results from the PT program. This is dependent upon (i) specimen preparation, (ii) storage conditions during handling and transportation, and (iii) use of specimens approximating those from patients. A stable PT specimen will ensure reproducibility of the virus-specific gene testing performed by the participating laboratories. The feasibility of using *in vitro* RNA transcripts as simulated specimens in EBOV PT panels was demonstrated here, and the stability of the simulated EBOV specimen was not compromised following long-term (1-year at −80°C) and short-term (a week at ambient temperature, followed by another week at −80°C) storage. Storage conditions for samples during handling and transportation may affect the stability of the target nucleic acid and therefore its detection. Of particular concern is whether sample storage reduces the viral load in samples with low virus concentrations, resulting in false-negative results (14, 15). Studies investigating the stability of EBOV RNA over time in collected samples reported that EBOV RNA is stable for 5 to 14 days under various conditions, i.e., dried onto solid surfaces or kept in the dark (16, 17) or in blood under simulated tropical conditions (18).

To our knowledge, the stability of lyophilized *in vitro*-transcribed RNA has not been reported elsewhere. Here, the simulated EBOV specimens containing *in vitro* RNA transcripts were stable upon lyophilization with the addition of RNAsecure reagent, which inactivates RNases and subsequent storage conditions (a total of 2 weeks of storage). These were designed to resemble the temperature (ambient) and maximum time required (1 week) during transport of the specimen, as offered in the third EBOV PT to participating international laboratories, as well as the maximum duration of time (2 weeks) given to participating laboratories to complete the EBOV PT. A previous study reported that lyophilized RNA virus (five different strains of influenza virus) was found to be more stable than fluid virus preparation indefinitely at or below room temperature (19). A separate study also reported that lyophilization of another RNA virus (human coronavirus [HCoV] NL63) preserved the virus at ambient temperature or at +4°C (20). Our finding suggests that in the absence of suitable samples (EBOV-positive clinical samples) as appropriate PT specimens, a simulated EBOV specimen can still be prepared using EBOV-specific *in vitro* RNA transcripts, either delivered on dry ice or lyophilized and delivered at ambient temperature. In particular, lyophilized *in vitro* RNA transcripts offer an advantage in PT programs, as they only require delivery to participating laboratories at ambient temperature, making them more cost-effective.

The use of the EBOV-specific *in vitro* RNA transcripts in the PT panel demonstrates the value of the simulated specimens in the absence of safe clinical specimens, as it is safe to handle, stable, and reliable. These simulated EBOV specimens are suitable as control material for development of PCR-based NAT assays by laboratories without preexisting capacity for EBOV detection. Similar approaches can be used in molecular assays for the detection of other high-risk organisms, and this represents a significant step forward in preventing harm from biothreats.

MATERIALS AND METHODS

Design of *in vitro* RNA transcripts. A comprehensive review of 18 published NAT-based EBOV detection assays was performed in late 2013, and the sequences of the primers used in these assays were collated to identify the most frequently used EBOV-specific gene targets. The EBOV gene fragments were determined using the complete genome sequence of EBOV (GenBank accession number AF086833.2) as a reference (Fig. 2). The fragments consisted of the following gene regions: a 1,406-nucleotide region upstream of the NP gene covering nucleotides at positions 518 to 1923, 500 nucleotides of the first GP gene at positions 7361 to 7860, a second GP gene region consisting of 873 nucleotides starting at position 6348, and a 750-nucleotide L gene starting at position 12885. These fragments were synthesized into a series of four double-stranded, sequence-verified genomic blocks (gBlocks gene fragments; Integrated DNA Technologies).

First, a residual adenine residue was added to the 3′ end of each individual gBlocks gene fragment using the activity of *Taq* DNA polymerase, thereby making the fragments compatible with T/A cloning vectors for cohesive-end cloning. Briefly, 50 ng of each individual gBlocks gene fragment was added to a single 15-μl total reaction mixture, including 1 to 3 units of *Taq* polymerase, 1× *Taq* polymerase buffer, 0.05 mM dATP, 1.5 mM $MgCl_2$, and nuclease-free water. The reaction mixture was then incubated at 70°C for 30 min. Two microliters from this reaction was then added into a 10-μl ligation reaction mixture, which consisted of 2 μl of 5× Express Link T4 DNA ligase buffer (Invitrogen), 1 μl of pCRII vector (25 ng/μl), 4 μl of nuclease-free water, and 1 μl of ExpressLink T4 DNA ligase (5 units), followed by

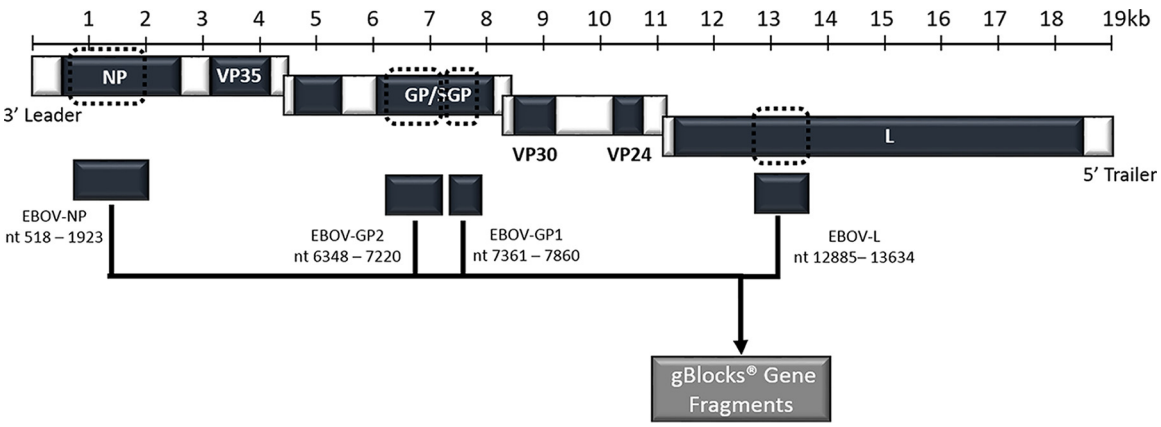

FIG 2 Schematic representation of the EBOV-specific gene fragments (NP, GP1, GP2, and L) used in the generation of *in vitro* RNA transcripts contained in the simulated EBOV specimens for the EBOV PT. The design of the EBOV gene fragments was based on the complete genome sequence of EBOV (GenBank accession number AF086833.2).

incubation at room temperature for 1 h. The ligated product was transformed into chemically competent *Escherichia coli* cells (α-Select Bronze Efficiency; Bioline) according to the manufacturer's protocol. The recombinant plasmid DNA was sequence verified and linearized using the restriction enzyme HindIII or XhoI for T7 RNA polymerase or SP6 RNA polymerase, respectively. Subsequently, the digested, linear plasmid DNA was transcribed *in vitro* into RNA the using MEGAscript T7 kit or MEGAscript SP6 kit (Ambion) as per the manufacturer's protocol. After DNase digestion and affinity purification, all four EBOV-specific RNA transcripts were quantified with a spectrophotometer.

Preparation of PT specimens. The simulated EBOV specimens were prepared by mixing each of the *in vitro* RNA transcripts, harboring parts of one fragment each of EBOV NP and L and two fragments of GP genes (EBOV GP1 and EBOV GP2). The mixture consisted of *in vitro* RNA transcripts in copy numbers that were similar to those found in clinical specimens (an average of approximately 1.0×10^8 genome equivalents [GE] copies/ml), with addition of 25× RNAsecure reagent (Ambion) and nuclease-free water to a total volume of 500 μl, and it was subsequently heated to 60°C for 10 min to eliminate RNase contamination. Prior to the distribution to participating laboratories, the simulated specimen was evaluated for its stability following long-term and short-term storage.

Evaluation of PT specimens. The simulated specimen was subjected to RNA extraction using the QIAamp viral RNA minikit (Qiagen) as per the manufacturer's protocol. Subsequently, the RNA was subjected to a one-step real-time RT-PCR. All one-step real-time RT-PCR mixtures were prepared as described here. A 25-μl RT-PCR mixture was set up, containing 5 μl of the extracted RNA, 12.5 μl of 2× reaction buffer provided with the AgPath-ID one-step RT-PCR reagents (Applied Biosystems), 1 μl of 25× RT-PCR enzyme mix (containing ArrayScript reverse transcriptase and AmpliTaq Gold DNA polymerase), 800 nM concentrations of forward and reverse primers, and 400 nM fluorogenic probes (all probes contained a 5′ fluorophore, FAM and double quenchers, an internal ZEN, and a 3′ BHQ1). All oligonucleotides were synthesized by Integrated DNA Technologies. Thermal cycling involved 45°C for 10 min, followed by 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min.

The long-term stability of the specimen was monitored in the following steps: (i) the simulated EBOV specimen was prepared in a replicate of 20 tubes, and five of the samples were randomly selected; (ii) these samples were then subjected to RNA extraction, as described above, prior to one-step real-time RT-PCR; and (iii) to assess the relative measure of the concentration of EBOV-specific target, quantification (GE copies/ml) or cycle threshold (C_T) was determined prior to storage using a set of EBOV-specific primers: EBOV-NP-Fwd (GCCAACGATGCTGTGATTTC), EBOV-NP-Rev (GGAGACGAACTCCTCGTTCTG), EBOV-GP1-Fwd (ACAAGTCCCCAAAACCACAG), EBOV-GP1-Rev (CTGCTGGCACTCTCTTCTCC), EBOV-GP2-Fwd (TGGGCTGAAAA/ideoxyI/TGCTACAATC), EBOV-GP2-Rev (CTTTGTG/ideoxyI/ACATA/ideoxyI/CGGCAC); EBOV-L-Fwd (GTTACGGAACGTGAGCAAAA), and EBOV-L-Rev (ACTGTGGCATGTTCACCAAA). The following fluorogenic probes were used: EBOV-NP-Prb (TGGCTCAAGCTCGTTTTTCAGGCTT), EBOV-GP1-Prb (CGCTG GCAACAACAACACTCATCAC), EBOV-GP2-Prb (CTACCAGCAGCGCCAGACGG), and EBOV-L-Prb (AAGCATC ATGGCACCACACAAGTGA). The remaining 15 samples were then stored at −80°C. One year later, another five samples were randomly selected, subjected to RNA extraction, and assessed for the C_T values poststorage, using the same set of EBOV-specific primers and probes described above.

The simulated EBOV specimen was also assessed for stability over a 14-day period. A set of 10 replicates of specimen was prepared (as described above), and all replicates were lyophilized for 20 h, as per the manufacturer's instructions (Lyoalfa 6 −80; Telstar). Three of the replicate lyophilized samples were randomly selected and were resuspended in 500 μl nuclease-free, sterile 1× phosphate buffered-saline (PBS). The material was subjected to RNA extraction and subsequent RT-PCR assessment (as described above). The remaining seven replicates were stored at ambient temperature (21 to 23°C) for a week, followed by 1 week of storage at −80°C. Following the 2-week storage, another three replicates of the simulated EBOV specimen were randomly selected, processed, and accessed in a similar way as for the initial three replicates prestorage.

The stability of the simulated EBOV specimen was determined by comparing the relative number of GE copies/ml of the specimen pre- and poststorage (calculated by dividing the GE copies/ml prestorage by the GE copies/ml poststorage), both on long-term storage (1 year at −80°C) and on short-term storage (2 weeks; 1 week at ambient temperature, followed by another week at −80°C). The significance of this comparison were determined using the two-tailed Mann-Whitney U test. The relative numbers of GE copies/ml in the specimen pre- and poststorage are shown as mean ± standard deviation (SD). Analyses were performed using GraphPad Prism v.6 (GraphPad, La Jolla, CA, USA). The differences of the relative numbers of GE copies/ml in the simulated EBOV specimen pre- and poststorage were considered significant when the *P* value was <0.05.

Participation and panel description. Australian laboratories were invited to participate in the EBOV PT offered in April 2014 and November 2014, with the invitation extended to international laboratories in March 2016. The majority of these were clinical diagnostic laboratories in government-funded hospitals, while some were national infectious diseases reference laboratories. Each round of PT (offered in April 2014, November 2014, and March 2016) consisted of two specimens, including one simulated EBOV specimen containing a mixture of EBOV-specific *in vitro* RNA transcripts (item 1) and a negative sample containing only nuclease-free water (item 2). Prior to the dispatch, all specimens were tested and confirmed for homogeneity based on five randomly selected samples. Specimens were provided either as 500 μl of the frozen form dispatched to the participating laboratories on dry ice (in April and November 2014) or in lyophilized form dispatched at ambient temperature (March 2016). The participating laboratories in the third round of PT (March 2016) were asked to resuspend lyophilized specimens in 500 μl of nuclease-free, sterile 1× phosphate buffered-saline (PBS). Laboratories were instructed to perform RNA extraction and subsequent NAT as they would normally do to identify the organism. Laboratories were requested to rule out or confirm the presence of EBOV and other filoviruses in all specimens of the PT panels. Correct responses for item 1 and item 2 were assigned on the basis of reporting the expected result, i.e., EBOV confirmed or ruled out and absence of other filoviruses. Each round of EBOV PT was closed 2 weeks following the dispatch.

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