The Journal of Infectious Diseases

SUPPLEMENT ARTICLE

Evaluation of RealStar Reverse Transcription–Polymerase Chain Reaction Kits for Filovirus Detection in the Laboratory and Field

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Background. Diagnosis of Ebola virus (EBOV) disease (EVD) requires laboratory testing.

Methods. The RealStar Filovirus Screen reverse transcription–polymerase chain reaction (RT-PCR) kit and the derived RealStar Zaire Ebolavirus RT-PCR kit were validated using in vitro transcripts, supernatant of infected cell cultures, and clinical specimens from patients with EVD.

Results. The Filovirus Screen kit detected EBOV, Sudan virus, Taï Forest virus, Bundibugyo virus, Reston virus, and Marburg virus and differentiated between the genera *Ebolavirus* and *Marburgvirus*. The amount of filovirus RNA that could be detected with a probability of 95% ranged from 11 to 67 RNA copies/reaction on a LightCycler 480 II. The Zaire Ebolavirus kit is based on the Filovirus Screen kit but was optimized for detection of EBOV. It has an improved signal-to-noise ratio at low EBOV RNA concentrations and is somewhat more sensitive than the Filovirus kit. Both kits show significantly lower analytical sensitivity on a SmartCycler II. Clinical evaluation revealed that the SmartCycler II, compared with other real-time PCR platforms, decreases the clinical sensitivity of the Filovirus Screen kit to diagnose EVD at an early stage.

Conclusions. The Filovirus Screen kit detects all human-pathogenic filoviruses with good analytical sensitivity if performed on an appropriate real-time PCR platform. High analytical sensitivity is important for early diagnosis of EVD.

Keywords. Filovirus; Ebola virus disease; commercial RT-PCR kit; molecular diagnostics; sensitivity.

The virus family *Filoviridae* contains 2 genera. The genus *Marburgvirus* consists of 1 species, *Marburg marburgvirus*, with the viruses Marburg (MARV) and Ravn (RAVV). The genus *Ebolavirus* contains 5 species: *Zaire ebolavirus* (Ebola virus [EBOV]), *Sudan ebolavirus* (Sudan virus [SUDV]), *Tai Forest ebolavirus* (Taï Forest virus [TAFV]), *Bundibugyo ebolavirus* (Bundibugyo virus [BDBV]), and *Reston ebolavirus* (Reston virus [RESTV]). The latter is endemic in Southeast Asia, while all other species are endemic in sub-Saharan Africa [1].

The signs and symptoms of EBOV disease (EVD), which may be caused by EBOV, SUDV, TAFV, and BDBV, and MARV disease are unspecific and resemble those of malaria, gastrointestinal infections, sepsis, and other viral hemorrhagic fevers. Therefore, diagnosis of acute EVD mainly relies on laboratory testing, specifically on reverse transcription–polymerase chain reaction (RT-PCR) analysis [2]. Assays for specific detection and differentiation of filovirus species [3–5], as well as broad-range assays for detection of various members of the family *Filoviridae* [6, 7], had been published before the West African EVD outbreak. During the outbreak, new commercial kits for nucleic acid or antigen detection of EBOV have been developed and validated [8]. In this study, we describe the RealStar Filovirus Screen RT-PCR Kit 1.0, which is designed to detect a broad range of filoviruses, including EBOV, SUDV, TAFV, BDBV, RESTV, MARV, and RAVV, as well as the RealStar Zaire Ebolavirus RT-PCR Kit 1.0. The latter is based on the former but has been optimized for EBOV detection. The European Mobile Laboratory (EMLab) used both kits for EBOV diagnostic testing in the field during the West African EVD outbreak. We report on the performance of the kits on various real-time PCR platforms.

MATERIALS AND METHODS

RealStar Filovirus Screen and Zaire Ebolavirus RT-PCR Kits

The RealStar Filovirus Screen kit (altona Diagnostics, Hamburg, Germany) targets a conserved region in the L gene [6]. Filovirus sequences available in GenBank as of December 2013 were used to design primers and probes able to detect EBOV, SUDV, TAFV, BDBV, RESTV, MARV, and RAVV. The reaction mix contains an internal control system amplifying a heterologous target sequence to monitor the purification procedure and check for inhibition of the RT-PCR. *Ebolavirus* species are detected in the FAM channel, *Marburgvirus* species in the Cy5

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The Journal of Infectious Diseases® 2016;214(S3):S243–9

DOI: 10.1093/infdis/jiw246

channel, and the internal control in the JOE channel. The RT-PCR conditions were optimized by titration of the PCR reagents. The RealStar Zaire Ebolavirus RT-PCR kit 1.0 (altona Diagnostics) is based on the Filovirus Screen kit but contains only those primers and probes required for EBOV detection. Details of the assay compositions are confidential intellectual property of altona Diagnostics.

Nucleic Acid Extraction and Cycling Conditions

RNA was extracted from 140 µL of plasma collected in tubes containing ethylenediaminetetraacetic acid, from 140 µL of cell culture supernatant, or from 50 µL of whole blood, using the QIAamp Viral RNA Mini kit (Qiagen) according to the manufacturer's instructions. Before extraction, 6 µL (one tenth of the elution volume) of the internal control template was added to the sample. The elution volume was 60 µL. The RT-PCR assay contained 10 µL of RNA and 20 µL of master mix from the RealStar kits. Thermal cycling comprised reverse transcription at 55°C for 20 minutes; activation of Taq polymerase at 95°C for 2 minutes; and 45 cycles at 95°C for 15 seconds, 58°C for 45 seconds with fluorescence acquisition, and 72°C for 15 seconds. Reactions were performed on LightCycler 480 II (Roche), CFX96 (Bio-Rad), Rotor-Gene Q and 6000 (Qiagen), and SmartCycler II (Cepheid) real-time PCR instruments with the same protocol.

Reference Filovirus RT-PCR Assay

Pan-filovirus primers and probes targeting the L gene, published by Panning et al [6], were used in conjunction with the AgPath-ID One-Step RT-PCR reagents (Life Technologies) as recommended by the German National Laboratory Network for Detection of Biological Threat Agents (NaLaDiBA). In brief, the 25-µL assay (also known as the Panning 2007 assay) contained 12.5 µL of buffer RT, 1 µL of enhancer, 1 µL of enzyme mix, 3 µL of RNA, 0.2 µM FiloA2.4, 0.2 µM FiloA2.2, 0.2 µM FiloA2.3, 0.3 µM FiloB, 0.3 µM FiloB-Ra, 0.08 µM FAMEBOSu, 0.08 µM FAMEBOg, and 0.08 µM FAMMBG. The reference assay was performed on the LightCycler 480 II instrument.

Reactivity, Sensitivity, and Specificity Testing

In vitro transcripts of the target sequences of EBOV Mayinga, EBOV Gabon 2003, EBOV Makona, SUDV Gulu, TAFV, RESTV, BDBV, MARV Popp, and MARV Leiden 2008 were generated using the MEGAScript T7 kit (Life Technologies) and purified using the QIAamp RNA Mini Kit, and the concentration was measured photometrically. Quantified in vitro transcript was used for determination of the 95% limit of detection (LoD95). To this end, the in vitro transcript was diluted in half-logarithmic steps in AE buffer (Qiagen) containing 10 µg/mL poly(A) RNA (GE Healthcare). The dilutions were directly tested in the RT-PCR assay in 8–12 replicates. The number of positive results per number tested (ie, the hit ratio) was subjected to probit analysis, using PriProbit, version 1.63 [9].

Assay reactivity was validated with RNA extracted from supernatant of cell-cultures infected with EBOV strains Mayinga, Gabon 2003, and Makona; SUDV strains Gulu and Maridi; RESTV; TAFV; and MARV strains Leiden 2008, Musoke, and Popp. Cross-reactivity of the assay was validated with clinical or cultured material containing the following pathogens: Japanese encephalitis virus, Saint Louis encephalitis virus, West Nile virus NY99, West Nile virus Uganda, yellow fever virus 17D, yellow fever virus French neurotropic vaccine, Murray Valley encephalitis virus, Zika virus, tick-borne encephalitis virus, Usutu virus, dengue virus 1, dengue virus 2, dengue virus 3, dengue virus 4, hepatitis C virus 3a, hepatitis C virus 1b, hepatitis A virus 1b, hepatitis E virus gg3c, CCHFV Afg09-2990, Lassa virus Nig08-A37, Lassa virus CSF, Lassa virus Lib05-1580/121, Lassa virus AV, Junin virus XJ, Machupo virus Carvallo, Sabia virus SPH114202, Guanarito virus INH-95551, vesicular stomatitis virus Indiana, Rift Valley fever virus MP12, and Hantaan virus 76-118. Thirty-six plasma samples from European blood donors were assayed with the Filovirus Screen kit to test for undesired cross-reactivity with human nucleic acid and for stable detection of the internal control. The Zaire Ebolavirus kit was not tested for cross-reactivity as it contains the same oligonucleotides as the Filovirus Screen kit. All reactivity and cross-reactivity data were generated using a LightCycler 480 II instrument.

External Quality Assessment

In March 2015, the EMLab unit in Coyah, Guinea, participated in an external quality assessment for EBOV RT-PCR field diagnostic testing organized by the Centers for Disease Control and Prevention (Atlanta, Georgia). Samples 1–5 were resuspended in 200 µL of water and extracted according to the protocol described above. From the 60 µL, 10 µL were used for RT-PCR. RNA samples 6–10 were resuspended in 40 µL of water, and 10 µL were used for RT-PCR. All samples were tested with both RealStar kits on Rotor-Gene and SmartCycler II instruments.

Retesting of Field Samples From Guéckédou

Specimens tested by the EMLab unit in Guéckédou were retrospectively retested in Hamburg to evaluate the clinical sensitivity of the kits. Samples were selected for retesting if they had been tested using the Filovirus Screen kit on a SmartCycler II in the field and if the patient had negative test results for 1 or several early samples but had a follow-up sample that tested positive for EBOV RNA. The extracted RNA of early and late samples was retested by using the RealStar kits on Rotor-Gene and CFX96.

Ethics

The National Committee of Ethics in Medical Research of Guinea, as well as the Ethics Committee of the Medical Association of Hamburg, approved the use of diagnostic leftover samples and corresponding patient data for this study (permit numbers 11/CNERS/14 and PV4910).

RESULTS

The design of the RealStar Filovirus Screen kit is based on the Panning 2007 assay [6], although primers and probes were modified to improve performance. The reactivity of the assay was validated with RNA from supernatant of cell cultures infected with EBOV strains Mayinga, Gabon 2003, and Makona; SUDV strains Gulu and Maridi; RESTV; TAFV; and MARV strains Leiden 2008, Musoke, and Popp; in vitro transcript was used for BDBV. The Filovirus Screen kit detected all *Ebolavirus* and *Marburgvirus* strains in the FAM and Cy5 channels, respectively, as expected. No cross-reactivity with nucleic acid in 36 human plasma samples or 30 human-pathogenic viruses (see Materials and Methods) was observed.

The sensitivity of the Filovirus Screen kit was approximated by comparing it against the Panning 2007 reference assay. To this end, log-scale dilutions of cell-culture-derived virus were spiked in human plasma and tested in triplicates. Both assays detected the same end point dilution for EBOV Mayinga and Makona and SUDV Gulu, while the Filovirus Screen kit reached a 2-log higher endpoint dilution with MARV Leiden 2008.

The analytical sensitivity, defined as the amount of target RNA that can be detected with a probability of 95% (ie, the LoD95), was determined by testing dilutions of in vitro transcript for SUDV Gulu, MARV Popp and Musoke, BDBV, and EBOV Gabon 2003 and Makona in 8–12 replicates, followed by probit analysis of the hit ratios. On the standard instruments recommended for the assay, the Rotor-Gene 6000, LightCycler 480 II, and CFX96, the Filovirus Screen kit achieved the following LoD95 values: 1.9 RNA copies/µL of RNA eluate (95% confidence interval [CI], 1.1–3.3 RNA copies/µL of RNA eluate) for EBOV Gabon 2003, 1.1 (95% CI, 1.0–1.2) for EBOV 2014/Gueckedou-C05, 6.7 (95% CI, 4.2–24) for SUDV Gulu, 1.1 (95% CI, .22–11) for MARV Popp, 4.2 (95% CI, 1.9–18) for MARV Musoke, and 1.8 (95% CI, 1.6–2.1) for BDBV. Given extraction of RNA from 140 µL of body fluid, elution in 60 µL, and input of 10 µL of RNA per reaction, these LoD95 values correspond to 471–2871 RNA copies/mL plasma.

For EBOV diagnostic testing in the West African EVD outbreak, EMLab and other laboratories used the SmartCycler II real-time PCR instrument. It is composed of 16 individual cycling units, a unique feature that facilitates rapid testing of samples once they arrive in the laboratory, irrespective of whether other samples are already running on the instrument. This ensures a low turnaround time, a key indicator of laboratory performance. However, others and we noticed reduced sensitivity of the Filovirus Screen kit on that instrument in the field. Testing of EBOV Makona in vitro transcript and probit analysis confirmed the significantly reduced analytical sensitivity of the kit on the SmartCycler II (Table 1), with LoD95 values corresponding to 17 100 RNA copies/mL body fluid.

We have taken 2 measures to cope with this problem. First, EMLab installed Rotor-Gene instruments in the field, on which the Filovirus Screen kit shows good analytical sensitivity as tested with EBOV in vitro transcript (Table 1). In addition, altona Diagnostics developed the Zaire Ebolavirus kit, which is identical to the Filovirus Screen kit but includes only those primers and probes required for detection of EBOV. This improves the signal-to-noise ratio for EBOV, particularly for samples with low virus load (Figure 1), while it abolishes the option to detect other filovirus species. Reactivity of the Zaire Ebolavirus kit was validated with RNA from supernatant of cell cultures infected with EBOV strains Gabon 2003 and Makona, SUDV Gulu, RESTV, TAFV, and MARV Musoke, as well as in vitro transcript for BDBV. As expected, the kit detected only the EBOV strains and showed some cross-reactivity with BDBV and RESTV in the FAM channel; no signals were seen in the Cy5 channel. Analytical sensitivity as tested with EBOV in vitro transcript did not differ significantly from the LoD95 for the Filovirus Screen kit (Table 1).

A small-scale evaluation in the EMLab field unit of the Zaire Ebolavirus and Filovirus Screen kits on both the Rotor-Gene Q and SmartCycler II revealed a gain in sensitivity due to use of the Zaire Ebolavirus kit and the Rotor-Gene (Table 2). The EMLab results for the external quality assessment organized by the Centers for Disease Control and Prevention in March 2015 for field laboratories further confirmed the superiority of the Rotor-Gene platform (Table 3). While the cycle threshold values hardly differ across the various platforms and kits,

Table 1. Analytical Sensitivity of the RealStar Kits on the Rotor-Gene 6000 and the SmartCycler II Platforms

	Filovirus Screen RT-PCR Kit		Zaire Ebolavirus RT-PCR Kit	
Variable	Rotor-Gene	SmartCycler II	Rotor-Gene	SmartCycler II
IVT concentration, copies/µL[a]				
3162	. . .	8/8	. . .	12/12
1000	. . .	8/8	. . .	12/12
316	8/8	8/8	8/8	12/12
100	8/8	8/8	8/8	8/12
32	8/8	7/8	8/8	7/12
10	8/8	5/8	8/8	1/12
3.2	8/8	1/8	8/8	1/12
1	5/8	0/8	5/8	0/12
0.3	0/8	. . .	3/8	. . .
0.1	0/8	. . .	0/8	. . .
LoD95 (95% CI)[b]	1.1 (1.0–1.2)	40 (20–251)	2.4 (1.2–16)[c]	233 (121–852)

Data are no. of positive results/no. of replicates tested, unless otherwise indicated. Probit analysis was used to calculate the 95% limit of detection.

Abbreviations: CI, confidence interval; RT-PCR, reverse transcription–polymerase chain reaction.

[a] In vitro transcripts (IVTs) based on Ebola virus 2014/Gueckedou-C05 were used to determine the hit rates for given concentrations. A 10-µL RNA solution was used per RT-PCR assay.

[b] Analytical sensitivity, defined as the amount of target RNA that can be detected with a probability of 95% (LoD95). Values represent RNA copies/µL of RNA solution.

[c] Comparable analytical sensitivity of the Zaire Ebolavirus RT-PCR Kit was obtained by testing EBOV Gabon 2003 IVT on the CFX96 platform (1.9 RNA copies/µL [95% CI, 1.1–6.9 RNA copies/µL]).

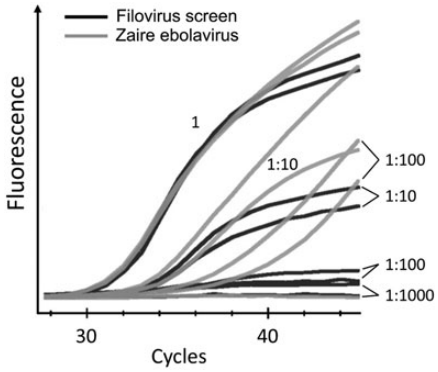

Figure 1. Fluorescence signal intensity determined by real-time reverse transcription–polymerase chain reaction analysis, using Zaire Ebolavirus and Filovirus Screen kits. Dilutions of Ebola virus (EBOV) RNA were assayed in parallel with Zaire Ebolavirus and Filovirus Screen kits on the CFX96 instrument. At low RNA concentrations, the fluorescence signal-to-noise ratio for the Zaire Ebolavirus kit is improved, compared with that for the Filovirus Screen kit.

indicating comparable amplification efficacy, the SmartCycler II–Filovirus Screen combination has difficulty detecting low concentrations of EBOV RNA corresponding to cycle threshold values of around ≥33 (Tables 2 and 3).

To evaluate whether this loss of sensitivity has clinical relevance, we retested samples that had been tested in Guéckédou by using the Filovirus Screen kit on the SmartCycler II. We selected all patients with EVD from the EMLab database (24 of 2741 individuals with suspected EVD from Guinea who have been tested in Guéckédou) whose initial sample(s) tested negative for EBOV by RT-PCR but had follow-up samples that tested positive for EBOV RNA. Retesting of the initially negative samples and the follow-up samples was performed using Zaire Ebolavirus and/or Filovirus Screen kits on Rotor-Gene and CFX96 platforms in Hamburg. For patients 1–10, the PCR results from the field were confirmed with all assays (Table 4). However, for patients 11–24, retesting revealed EBOV RNA in several early samples that had negative or inconclusive test results in Guéckédou (Table 4). The Filovirus Screen kit on Rotor-Gene detected EBOV RNA in 8 additional samples, the Zaire Ebolavirus kit on Rotor-Gene detected EBOV RNA in 15 additional samples, and the Zaire Ebolavirus kit on CFX96 detected EBOV RNA in 14 additional samples. These data further confirm the gain in sensitivity that is provided by the Rotor-Gene and Zaire Ebolavirus kit. The median cycle threshold of samples, which were positive upon retesting with the Zaire Ebolavirus kit on Rotor-Gene, was 30.9 (range, 26.7–40.6). The 7 samples that tested positive by the Zaire Ebolavirus kit but negative by the Filovirus Screen kit on Rotor-Gene had a median cycle threshold of 37.8 (range, 30.9–40.6). These data indicate that enhancing sensitivity facilitates earlier detection of EVD after onset of disease.

Table 2. Relative Sensitivity of Filovirus Screen and Zaire Ebolavirus Kits on Different Polymerase Chain Reaction (PCR) Platforms as Evaluated With Ebola Virus (EBOV) RNA From Patient Samples in the EMLab Field Unit

	Rotor-Gene Q, Ct		SmartCycler II, Ct	
Sample No., RNA Dilution[a]	Filovirus Screen Kit	Zaire Ebolavirus Kit	Filovirus Screen Kit	Zaire Ebolavirus Kit
1 (15.5)				
10^{-1}	18.5	18.1	18.6	18.9
10^{-2}	23.2	21.7	22.3	22.6
10^{-4}	30.0	28.7	29.1	29.6
10^{-5}	33.7	32.4	Negative	33.4
10^{-6}	Negative	37.1	Negative	36.4
2 (20.1)				
10^{-1}	22.9	22.8	22.5	23.4
10^{-2}	27.4	26.7	26.3	27.1
10^{-4}	35.0	34.9	35.8	33.6
10^{-5}	Negative	Negative	Negative	Negative
3 (31.1)				
10^{-1}	Negative	37.6	Negative	Negative
10^{-2}	Negative	37.7	Negative	Negative
10^{-3}	Negative	Negative	Negative	Negative

RNA from patient samples was extracted, and diagnostic EBOV reverse transcription–PCR was performed using the Filovirus Screen kit on the SmartCycler II in the EMLab field unit in Coyah, Guinea. For the evaluation, the stored RNA was diluted in log-steps and tested in parallel on the different platforms.

Abbreviations: Ct, cycle threshold; EMLab, European Mobile Laboratory.

[a] The Ct of the diagnostic PCR is given in parentheses.

Table 3. EMLab Results for the External Quality Assessment of Field Laboratories, Organized by the Centers for Disease Control and Prevention (CDC) in March 2015

	Rotor-Gene Q, Ct		SmartCycler II, Ct	
CDC Sample ID, Expected Result	Filovirus Screen Kit	Zaire Ebolavirus Kit	Filovirus Screen Kit	Zaire Ebolavirus Kit
1, negative	Negative	Negative	Negative	Negative
2, positive	32.3	32.0	30.9	31.9
3, positive	24.0	24.3	24.1	25.0
4, negative	Negative	Negative	Negative	Negative
5, positive	37.3	36.0	Negative[a]	Negative[a]
6, positive	22.2	21.7	21.9	22.0
7, negative	Negative	Negative	Negative	Negative
8, negative	Negative	Negative	Negative	Negative
9, negative	Negative	Negative	Negative	Negative
10, positive	28.7	29.2	28.4	28.9

Abbreviations: Ct, cycle threshold; EMLab, European Mobile Laboratory; ID, identifier.

[a] False-negative result.

DISCUSSION

In this study, we present analytical and clinical validation data for the RealStar Filovirus Screen and Zaire Ebolavirus RT-PCR kits, version 1.0. The Filovirus Screen kit detects all relevant filovirus species with high analytical sensitivity on the recommended real-time PCR platforms. The Zaire Ebolavirus kit has been optimized for detection of EBOV. It shows comparable

Table 4. Retesting of Samples From Patients With Ebola Virus (EBOV) Disease (EVD) Diagnosed by the EMLab in Guéckédou Whose Initial Specimen Tested Negative for EBOV RNA but Had a Follow-up Specimen That Tested Positive

Patient No., Days After Onset[a]	SmartCycler II Result of EMLab Field Unit Using the Filovirus Screen Kit, Ct	Retesting on Rotor-Gene 6000, Ct		Retesting on CFX96 Using the Zaire Ebolavirus Kit, Ct
		Filovirus Screen Kit	Zaire Ebolavirus Kit	
1				
3	Negative	Negative	Negative	Negative
10	27.3	27.6	26.6	27.5
2				
3	Negative	Negative	Negative	Negative
5	29.1	29.7	28.4	29.0
3				
4	Negative	Negative	Negative	Negative
14	28.4	28.9	27.7	28.1
4				
5	Negative	Negative	Negative	Negative
13	13.9	13.6	13.4	12.7
5				
6	Negative	Negative	Negative	Negative
7	27.7	26.8	26.0	27.1
6				
7	Negative	Negative	Negative	Negative
8	25.2	ND	ND	ND
7				
1	Negative	Negative	Negative	Negative
3	20.3	20.9	19.5	20.3
8				
16	Negative	Negative	Negative	Negative
21	18.1	18.4	17.1	17.9
9				
4	Negative	Negative	Negative	Negative
12	19.5	19.8	18.4	19.2
10				
5	Negative	Negative	Negative	Negative
6	Negative	Negative	Negative	Negative
16	15.9	16.4	14.7	15.7
11				
1	Negative	Negative	Negative	Negative
3	Negative	Negative	Negative	Negative
5	**Inconclusive**	**30.8**	**29.5**	**30.7**
6	20.7	21.4	19.9	20.7
12				
4	Negative	**27.9**	**26.7**	**27.5**
5	28.3	29.5	28.2	29.2
13				
6	Negative	**29.9**	**28.1**	**28.8**
11	17.9	16.9	15.6	16.1
14				
2	Negative	**29.0**	**29.7**	**29.5**
4	24.7	24.7	23.9	24.6
15				
1	Negative	**34.0**	**30.4**	**30.8**
3	21.4	20.5	19.2	19.7
16				
0	Negative	**33.0**	**29.3**	**30.1**
3	15.4	16.2	14.5	15.2
17				
0	Negative	**34.1**	**30.9**	**31.2**
1	23.1	23.4	22.2	22.9
18				
5	Negative	Negative	**38.7**	**39.5**
8	22.3	21.3	20.2	21.3
19				
4	Negative	Negative	**33.4**	**33.2**
10	24.4	28.9	25.3	25.8
20				
1	Negative	Negative	**40.6**	**42.7**
4	Negative	**33.8**	**32.5**	**34.4**
5	18.2	18.4	16.8	18.0
21				
7	Negative	Negative	**32.2**	**32.5**
9	29.5	29.8	29.4	29.2
22				
1	Negative	Negative	Negative	Negative
3	Negative	Negative	**30.9**	**31.4**
4	27.7	28.9	ND	28.7
23				
1	Negative	Negative	Negative	Negative
3	Negative	Negative	**37.8**	**39.8**
4	30.2	34.5	36.4	31.9
24				
9	Negative	Negative	**40.6**	Negative
14	22.3	21.7	20.3	21.3

Extracted RNA from early and late samples of these patients was retested in Hamburg using different real-time PCR platforms and kits. Discrepant results are marked in bold.

Abbreviations: EMLab, European Mobile Laboratory; ND, not done due to insufficient leftover RNA.

[a] These data must be interpreted with caution. This information was taken from the World Health Organization database and sometimes differed from the information provided on the EMLab request forms. However, the time between collection of the 2 samples is based on the date of sampling recorded in the EMLab database.

analytical data for this species and improved clinical sensitivity in the early phase of EVD. Both kits are significantly less sensitive on the SmartCycler II.

The EMLab started diagnostic service in Guéckédou in March 2014. The established work flow of the mobile unit included sample inactivation in a glove box, manual nucleic acid extraction by using the QIAamp viral RNA kit, and real-time RT-PCR on the SmartCycler II to ensure a quick turnaround [10]. Before field deployment, EMLab compared in-house assays available at that time for EBOV diagnostic testing, such as the Panning 2007 pan-filovirus assay [6] and the EBOV/SUDV-specific assay published by Gibb et al in 2001 [4], to the prototype of the Filovirus Screen kit. Because the kit outperformed the in-house assays on the EMLab platform, it was chosen for the mission. In addition, the Filovirus Screen kit

provided the advantages of easy reaction set up, minimizing potential pipetting errors, quality-assured performance and reagents, and internal control system for monitoring the whole process. The latter feature has been crucial in the field to identify false-negative reactions, which were often observed with swab samples from human bodies (potentially due to prior treatment of the sampling sites with bleach that thus entered the reaction). The Filovirus Screen kit was eventually launched in April 2014 for research use only and in September 2014 as a Conformité Européene–marked in vitro diagnostic product.

At the end of 2014, others and we noticed the reduced sensitivity of the kit on the SmartCycler II as compared to alternative instruments [11–14]. We should mention that the Filovirus Screen kit has been optimized for use on other real-time PCR platforms, such as the LightCycler 480 II or CFX96, while the SmartCycler II is not recommended, according to labeling and manufacturer's instructions [15]. A lesson learned from our findings is that performance of PCR may be significantly affected by the real-time PCR instrument and must be verified for each platform. On the other hand, the Filovirus Screen kit has been used successfully by others on instruments qualified by altona Diagnostics, with good sensitivity and reliable results [16–18]. A recent study compared different methods analytically and found the Filovirus Screen assay to be comparable in sensitivity to other commercial assays for detection of EBOV; only the Lifetech Lyophilized Ebola Virus (Zaire 2014) kit (Life Technologies) showed better sensitivity than all other commercial kits validated [19]. The analytical sensitivity obtained in this external validation study (1250 EBOV RNA copies/mL) corresponds quite well with the analytical data we present here for recommended cycler types. However, compared to other commercial kits, the Filovirus Screen assay facilitates detection of all human-pathogenic filovirus species and RESTV.

We reasoned that performance of the Filovirus Screen kit for EBOV detection could be improved by omitting all primers and probes that are not essential for detection of this particular species. This might reduce undesired cross-reactivity and interference between primers and probes, and therefore it was plausible to assume that sensitivity and specificity increase rather than decrease. From a technical point of view, such modification can be more easily implemented than designing a new assay. Indeed, focusing the kit on EBOV detection improved the signal-to-noise ratio at low RNA concentrations. While the probit analysis could not demonstrate an effect, owing to wide 95% CIs, a small-scale comparison of both RealStar kits in the field indicated somewhat improved sensitivity. Based on these data, it was decided to use the Zaire Ebolavirus kit in the EMLab units, and altona Diagnostics provided the kit as a product for research use only from March 2015 onward. The retrospective clinical evaluation, using samples collected from patients early during EVD, eventually confirmed the improved sensitivity of the Zaire Ebolavirus kit as compared to the source kit. Analytical and clinical data also indicate that replacement of the SmartCycler II by the Rotor-Gene in the field led to a substantial increase in sensitivity.

The retrospective testing of early samples from patients with EVD revealed that high sensitivity is important for early diagnosis. While the patients retested here received their diagnosis in the field after analysis of follow-up samples, it might be that other patients who had a false-negative result for the initial sample did not return to the Ebola treatment unit for follow-up and thus escaped detection. Based on currently available data, it is not possible to estimate how many patients with EVD have been missed because of the reduced sensitivity of the SmartCycler II platform. Retesting samples from a representative set of patients with suspected EVD who tested negative and were classified as noncases may provide a clue. Now, highly sensitive technologies are available that can be used for this purpose. In addition, our most sensitive assay did not detect EBOV RNA in the first sample obtained from 13 of 24 patients with EVD. This suggests that, in a fraction of patients with EVD, the virus load is below the limit of detection of standard RT-PCR assays in the first days after onset of symptoms. The fraction of such patients is not known; the 24 cases presented here represent only 0.88% of all suspected cases of EVD tested by EMLab in Guéckédou. The 13 samples that initially tested negative were collected a median period of 4 days after onset, although data on the day of onset data are not reliable, and coinfections, which may obscure the EVD onset, have not been ruled out. In any case, our data reinforce the existing recommendation to monitor and retest patients with suspected EVD for 72 h after onset of symptoms [20].

Notes

Acknowledgments. We thank the World Health Organization (WHO) field teams and the Guinean health authorities, for their commitment and excellent cooperation. The European Mobile Laboratory is a technical partner of the WHO Emerging and Dangerous Pathogens Laboratory Network and the Global Outbreak Alert and Response Network (GOARN), and the deployments in West Africa have been coordinated and supported by the GOARN Operational Support Team at WHO headquarters.

Financial support. This work was supported by the European Union's Horizon 2020 research and innovation program (grant 666100 to the EVIDENT [Ebola virus disease: correlates of protection, determinants of outcome, and clinical management] project), by the Directorate-General for International Cooperation and Development (service contract IFS/2011/272-372), and the European Fund for Regional Development (EFRE project BWF/H/52228/2012/13.10.10-1/3.4,6).

Potential conflicts of interest. S. Ö. and H. E. H. are employees of altona Diagnostics. They are not shareholders. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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