Diagnostic Reverse-Transcription Polymerase Chain Reaction Kit for Filoviruses Based on the Strain Collections of all European Biosafety Level 4 Laboratories

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A network of European biosafety level 4 laboratories has designed the first industry-standard molecular assay for all filoviruses species, based on the strain collections of all participants. It uses 5 optimized L gene primers and 3 probes, as well as an internal control with a separate detection probe. Detection limits (probit analysis, 95% detection chance) were as follows: Zaire ebolavirus, 487 copies/mL of plasma; Sudan ebolavirus Maleo, 586 copies/mL; Sudan ebolavirus Gulu, 1128 copies/mL; Cote d’Ivoire ebolavirus, 537 copies/mL; Reston ebolavirus, 4546 copies/mL; Lake Victoria marburgivirus Musoke, 860 copies/mL; and Lake Victoria marburgivirus Ravn, 1551 copies/mL. The assay facilitates reliable detection or exclusion screening of filovirus infections.

The family Filoviridae in the order Mononegavirales contains 2 genera of pathogenic viruses, Ebolavirus (EBOV) and Marburgvirus (MARV). EBOV comprises the species Zaire ebolavirus (ZEBOV), Sudan ebolavirus (SEBOV), Cote d’Ivoire ebolavirus (CIEBOV), and Reston ebolavirus (REBOV). As agents of human disease, filoviruses are usually encountered in the context of outbreaks in Africa. Imported cases of filovirus infection are very rare. Nevertheless, potential importation of filoviruses by returning travelers is a frequent concern because of symptom similarity with other diseases—for example, malaria—and the risk of nosocomial filovirus transmission. Although, in a confirmed outbreak situation, case definitions can partially substitute for laboratory tests [1], in suspected cases of importation, laboratory diagnosis is always required. Severely ill patients have to be tested for a number of hemorrhagic fever viruses (e.g., EBOV, MARV, Lassa virus, yellow fever virus, and Crimean-Congo hemorrhagic fever virus) in a very short time and with such high reliability that an excluding result can be issued [2]. Similar scenarios arise in the screening of sick nonhuman primates, in the monitoring of workers after laboratory accidents, and in the context of suspected bioterrorism.

Reliable methodology can be established only if appropriate virus material is available. However, virus strains and clinical samples are limited and are subjected to biosecurity regulations. Polymerase chain reaction (PCR) design often relies on theoretical genome information only, which, for filoviruses, is incomplete in public databases. A recent external quality-assurance study has demonstrated that the overall level of diag-

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nositic PCR proficiency for filoviruses is low [3]. Real-time PCR, especially ready-to-use test kits, could improve this situation, but such reagents are not available from the industry.

Within a network supported by the European Union, all European biosafety level 4 (BSL4) laboratories, together with a biotechnology company (Qiagen), have developed a diagnostic real-time PCR assay for filoviruses. Strains stored in the European BSL4 laboratories were collated and sequenced, an assay reacting with all filoviruses was designed, and the assay was evaluated with representative filoviruses. The test was then converted into a prototype test kit. We present here the in-house version of the test, as well as comprehensive evaluation data for the prototype kit.

**MATERIALS AND METHODS**

Filovirus strains were grown in Vero E6 cells under BSL4 conditions. The virus titer in supernatant was determined by immunological focus assay using EBOV- or MARV-specific antibodies. Parts of the L genes of ZEBOV Eckron (BSL4 laboratory in Marburg, Germany), Gabon 1995 (Lyon, France), Gabon 2001 (Lyon, France), and Gabon 2003 (Hamburg, Germany) were amplified and sequenced using primers EboZ-13070+ (AATGGCTAGGTATACTCCACCA), EboZ-13126+ (ACTAAGGTGTAACCGGAAAATTT), EboZ-13720— (TGTTGAGGATTGACCCAGTATG), and EboZ-13760— (ACTAAAGAATTTGAGACACATGA). Parts of the L gene of SEBOV Gulu (Hamburg), CIEBOV (Marburg), and MARV Ravn (Stockholm) were amplified and sequenced using primers EBO-12988+ (TTTCACACAAATTTATWGTGTA), EBO-13007+ (AGTTAGTATTTAAGYATYTTCATAAARGA), EBO-13752— (ATTTGAGACAGAATATACGTCCA), EBO-13824— (ACGGTTATACAYTGATCTACCCAT), and EBO-13980— (TAGTGAACACTGARTGKACRAATGT).

The initial assay design used the new sequences and published data of ZEBOV Zaire95 (GenBank accession no. AY354458) and Mayinga (AY142960); SEBOV Maleo (EU-23458); REBOV Reston (AB050936) and Pennsylvania (AF-522874); and MARV Popp (MVVRPR), Ozolin (AY-358025), and Musoke (MRVMBGL). On inspection of the alignment, relatively conserved binding sites for primers and probes were avoided to guarantee reliable resynthesis of primers. When necessary, virus sequence heterogeneity was compensated for by mixing of defined oligonucleotides. The stable non–Watson-Crick base pairing T:G was not strictly adjusted for.

Quantified in vitro transcribed RNA was generated by cloning the reverse-transcription (RT) PCR target region, together with sufficient flanking sequence, in plasmid pCR2.1. Inserts, together with a 5’-T promoter sequence, were amplified with M13 plasmid-specific primers and transcribed in vitro into RNA by use of the Megascript T7 kit (Ambion). After DNase digestion and affinity purification, transcripts were quantified by photometer and used as quantification standards.

To monitor the sensitivity in each single reaction of the assay, an internal competitive control was constructed. It consisted of a copy of the target RNA sequence, containing a mutagenized probe binding site that could be detected by an alternative probe, which was labeled with its own specific reporter dye. The control was generated by overlap-extension PCR on the ZEBOV plasmid, as described elsewhere [4], using EboZ-13126+/EboZ-13720— and mutagenic primers ATCGTGTTGAGGATTGACCCAGTATG and CTGCTAACTCGCTCAACGAACGATTGTCCATGTAGACAAATTTGAGACACATGA. Mutated constructs were cloned back into pCR2.1 and transcribed into RNA.

The in-house version of the diagnostic assay was based on the Qiagen 1-step RT-PCR kit, using a 25-μL total reaction volume including 3 μL of RNA from the Qiagen viral RNA mini kit. Reactions were supplemented with 40 ng/μL bovine serum albumin and 400 μM dNTP. The primers used were as follows: FiloA2.4, AAGCATTTTCTAGCAATATGATTGGGT (200 nmol/L); FiloA2.5, AAGGCTTTTCTACTGAACATAGTGGTGTT (200 nmol/L); FiloA2.6, AAGGCTTTTCTACTGAACATAGTGGTGTT (200 nmol/L); and Filo B-Ra, GTGGAGGTGGCTGAGTGCTCGAATG (300 nmol/L). Probes included FAMEBO5u, FAM-CCGAACATCAGCTTTTGTTGCGGCA-BlackHole Quencher 1 (BHQ1) (66.7 nmol/L); FAMEBO7g; FAM-CCGAAATCTACTGCTTTTGTTGCGGCA-BHQ1 (66.7 nmol/L); FAMEBO5u, FAM-CCGAACATCAGCTTTTGTTGCGGCA-BlackHole Quencher 1 (BHQ1) (66.7 nmol/L); FAMEBO5u, FAM-CCGAACATCAGCTTTTGTTGCGGCA-BlackHole Quencher 1 (BHQ1) (66.7 nmol/L); and YFPR, DyXL-ATCGTTGCTTGGAGCGATTAGCAG-BHQ1. All probes were synthesized by Tib-Molbiol (Berlin, Germany). Amplification in a Roche Light Cycler 1.2 involved the following steps: 50°C for 30 min; 95°C for 15 min; and 45 × 95°C for 15 s, 52°C for 25 s, and 72°C for 20 s. Fluorescence was measured in each cycle at the 52°C step. The virus signal was read at the F1 wavelength, and the internal control signal was read at the F2 wavelength.

**RESULTS**

The L gene of filoviruses was selected because a pair of primers targeting this gene (Filo-A and Filo-B) has been used successfully in a number of previous studies [5–9]. Seven new sequences for the target region were generated (GenBank accession no. EF490228, SEBOV Gulu 10/200; EF490229, ZEBOV Gabon 1995; EF490230, ZEBOV Gabon 2003; EF490231,
Figure 1. Design of the in-house assay serving as a basis for the test kit. A, Binding sites of oligonucleotides. Letters in the left column represent the species (Z, Zaire ebolavirus [ZEBOV]; IC, Cote d’Ivoire ebolavirus [CIEBOV]; RE, Reston ebolavirus [REBOV]; S, Sudan ebolavirus [SEBOV]; and M, Lake Victoria marburgvirus [MARV]), followed by a representative strain designation (GenBank accession nos. or sequences from in-house strain collections). Note that only representative sequences are shown. All sequences occurring more than once at each of the oligonucleotide binding sites have been deleted from the alignment. The alignment was updated in October 2006 with latest GenBank entries. The complete alignment can be retrieved from http://www.bni-hamburg.de/ebovalign. B, A probe matching ZEBOV (top panel), modified to adapt a single nucleotide mismatch as present in REBOV (probe EBOg, lower panel). No loss of signal occurred for ZEBOV (bottom panel), and the signal for REBOV improved (not shown; REBOV was not yet available at the time these experiments were performed). C, top panel, A probe containing 2 inosin residues at positions of variability but otherwise matching SEBOV (probe EBOSu), yielding a good detection signal (similar to the signal from a corresponding RNA concentration of ZEBOV; compare with panel B). C, middle panel, A probe of similar design, adapted to CIEBOV, yielding good signal for CIEBOV. However, because CIEBOV was also detectable with good efficiency with the EBOSu probe (C, bottom panel), EBOSu was used for both SEBOV and CIEBOV. D, Detection signals with oligonucleotides, as shown in panel A, in reactions containing low concentrations of virus RNA (flat lines denote controls; the figure shows signals with all probes and primers mixed in 1 reaction). E, Determination of working concentration for competitive internal control. A constant small amount of target gene RNA (solid line; 50 copies of ZEBOV transcript per assay) was amplified in the presence of increasing concentrations, as plotted on the X-axis, of the internal control (dotted line). Observed crossing point values were plotted on the Y-axis for both target genes (note that the virus and internal control carried different reporter dyes). Increasing values on the Y-axis indicate decreasing amplification efficiencies. From 40 copies of internal control onward, stable amplification was possible. From 400 copies/reaction (cps/rxn) onward, amplification of ZEBOV RNA lost efficiency, as is represented by the increasing crossing point value.
Figure 2. Performance of the ready-made filovirus test kit. A, Testing of plaque-quantified virus stock solutions. Virus was diluted in virus-negative human serum to the listed concentrations and tested in 4 replicate assays each. ++, positive result; --, negative result; RT-PCR, reverse-transcription polymerase chain reaction. B, Probit analysis on replicated tests (typically 6 tests per concentration) of virus-negative human serum inoculated on the level of the lysis buffer with given concentrations (X-axis) of filovirus RNA in vitro transcripts. The observed rates of positive results per total no. of tests performed are plotted on the Y-axis. Nos. in each panel are the concentrations beyond which the statistical chance of detection exceeds 95%, according to the probit model (dose-response relationship). Nos. in parenthesis are 95% confidence intervals for these numbers. cps, copies.
Gulu and MARV Musoke. SEBOV was detected with in vitro transcribed RNA of SEBOV by probit analyses, as described elsewhere [5, 12]. These experiments used quantified in vitro transcribed RNA of SEBOV, EBOV, and MARV, as shown in figure 1A. With inosine-containing probes, it was possible to compensate for as many as 4 nucleotide mismatches, as maximally observed in EBOV, without losing signal intensity (figure 1B and 1C). For MARV, such an adaptation was not necessary. There was no need to change the assay design in view of more recently published filovirus sequences [10]. Figure 1A provides a summary of sequence diversity observed at all oligonucleotide binding sites, as updated in October 2006. Sequences from the 2005 outbreak of MARV infection in Uige, Angola, were not yet published when oligonucleotides were designed for this study, but compatibility with these sequences was confirmed to us before publication [11].

The mixing of detection probes for EBOV and MARV in 1 reaction led to a slight decrease in plateau fluorescence signal, as opposed to that seen with separate detection. However, it did not affect the overall assay sensitivity. As shown in figure 1D, RNA of all tested strains was detectable at high sensitivity, ~10 copies/assay. Primer and probe combinations were tested on genomic RNA extracted from virus preparations. Amounts of ∼0.1 pfu of ZEBOV, SEBOV, or MARV could be detected per assay. The assay was now supplemented with a competitive internal control, for which an optimal working concentration of 50 copies/assay was determined by cross-titration against a constant low amount of target RNA (figure 1). The limit of detection of the in-house assay was determined by probit analyses, as described elsewhere [5, 12]. These experiments used quantified in vitro transcribed RNA of SEBOV Gulu and MARV Musoke. SEBOV was detected with a >95% chance if plasma contained at least 1144 RNA copies/mL (95% confidence interval [CI], 826–4088 copies/mL). The corresponding number for MARV Musoke was 1508 copies/mL (95% CI, 958–5251 copies/mL).

Specificity for filoviruses was confirmed using a panel of heterologous viruses. The assay did not cross-react with RNA extracted from undiluted cell culture supernatants of Lassa virus, Crimean-Congo hemorrhagic fever virus, dengue virus, Epstein-Barr virus, herpes simplex virus type 1, hepatitis C virus, HIV type 1, Japanese encephalitis virus, yellow fever virus, West Nile virus, polio virus, Ross River virus, Barmah Forest virus, Sindbis virus, and Venezuelan equine encephalitis virus.

The assay was transformed into a prototype “artus Filovirus LC RT-PCR Kit,” using proprietary technology, preserving oligonucleotides as described. A proprietary noncompetitive internal control RNA was included instead of the competitive internal control used in the in-house version. Sensitivity was evaluated on plaque-quantified virus stock material, as shown in figure 2, as well as on sample panels from a recent proficiency testing study [3]. All filovirus samples from this study were correctly detected, and all RNA quantification results showed <1 log10 deviation from predetermined data [3]. Clinical specimens were available from 2 patients: 1 serum sample from an outbreak of SEBOV infection in Uganda and 1 buccal swab from an outbreak of ZEBOV infection in Gabon. Testing of both materials by RT-PCR has been described elsewhere [1, 5]. Both were readily detected with the assay.

Intra- and interassay variability were also determined. Twenty parallel determinations in 1 run on a sample containing 1000 copies/reaction yielded an intra-assay coefficient of variation of 0.66% (based on an average crossing point value of 32.95). When the same sample was tested in 11 independent runs, an interassay coefficient of variation of 1% was seen at an average crossing point value of 32.65. The precise limits of detection were determined for a collection of virus strains representing the spectrum of genetic diversity at the oligonucleotide binding sites. Results of probit analyses are summarized in figure 2.

CONCLUSIONS

Recent studies of rare pathogens, such as severe acute respiratory syndrome (SARS) coronavirus, orthopox viruses, or West Nile virus, have shown that the availability of ready-made test kits positively influences laboratory performance [13–15]. The feasibility of developing such kits through close collaboration with industry has been demonstrated in connection with the SARS outbreak in 2003, when a test kit was made available during the epidemic. The main purpose of the present study was to design a similar kit for filoviruses. Most of the preliminary work was done by academic and governmental institutions, lowering the financial entry threshold for a company that collaborated as a network partner. Kits could be produced in accordance with high quality standards, with features comparable to those of commercial diagnostic PCR systems (e.g., HIV-1, hepatitis C virus, and hepatitis B virus). This is the first probe-based assay compatible with all known filovirus species. An internal control monitors the sensitivity in every single reaction. Analytical properties have been evaluated in accordance with industry-approved standards, such as probit analysis with a broad range of filovirus strains, intra- and inter-assay variability analysis, and specificity testing with an exhaustive panel of viruses causing related clinical manifestations. These
data not only will ensure wide acceptance of the assay as a standard test in Europe but could also serve as an entry point into production as an in vitro diagnostic procedure.

Irrespective of whether the assay is used in its in-house version or as a reagent kit, it cannot be guaranteed that a PCR assay will detect unknown filovirus strains that may emerge in the future. The diagnostics of filovirus infection should always be based on the whole range of diagnostic tests, including PCR, antigen testing, antibody detection, and virus culture. Moreover, new technical developments and novel virus strains impose continued work on molecular assays. The present study has shown that such work is possible only through collaboration and exchange of virus material. In view of expanding restrictions in the context of biosecurity, care has to be taken that such indispensable collaboration will still be possible in the future.

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