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Coordinated implementation of a conventional PCR assay to detect all Ebola and Marburg virus species in a European laboratory network

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ABSTRACT

Background: Filoviruses, including Ebola and Marburg viruses, cause severe hemorrhagic fever in humans and primates. These viruses pose significant threats to public health, making rapid and sensitive detection critical for controlling outbreaks. We developed and validated a hemi-nested generic PanFilo assay to detect all Ebola virus species, Marburg viruses, and recently discovered bat filoviruses. This assay was deployed to 15 European laboratories and evaluated through testing of eight non-infectious samples.

Objectives: Laboratories were asked to determine the detection limit of positive controls and test all samples using the assay provided. The deployed assay enables direct Nanopore sequencing of PCR products, by using tagged primers during the second round of PCR. Sequencing of the samples was carried out on a voluntary basis.

Results: Multicenter validation revealed a 95 % limit of detection of 5309 RNA copies/μL for Ebola, 10,273 copies/μL for Marburg, and 2145 copies/μL for Mengla virus. In an implementation quality assessment, 93.3 % (84/90) of samples containing filovirus RNA were correctly identified and 100 % (30/30) of filovirus-negative samples were correctly identified. Thirteen laboratories sequenced PCR products, with nine identifying all positive samples correctly.

Conclusion: The assay enables rapid and reliable detection of filoviruses, with sequencing capabilities for identifying both known and novel variants. This assay might be used for detection during the initial phase of an emerging filovirus outbreak, before a specific assay has been developed. However, our distribution across 15

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laboratories revealed variability challenges due to reagents, human performance, and sequencing capacity, emphasizing the need for more training and standardization.

1. Introduction

Human pathogenic filoviruses are endemic to western and equatorial Africa [1]. They can cause acute disease and hemorrhagic fever with high mortality [1,2]. Occasional imported cases have been reported in Europe and the Americas [3]. Their public health importance was highlighted by the 2013 West African Ebola virus (EboV) outbreak, resulting in 11,323 deaths [2,3]. As a result, EboV and Marburg virus (MarV) diseases are included in the WHO's Research and Development (R&D) Blueprint [4]. A critical problem at the beginning of an outbreak is the limited access to diagnostics, making early containment difficult [5]. Broadly reactive generic PCR assays can play an important role for emerging variants, before highly specific PCR assays are developed or widely available. Moreover, generic PCR can aid in identifying new variants when more specific routine tests are negative.

In this study, we describe a hemi-nested generic filovirus PCR assay designed to detect all EboV, MarV, and recently discovered bat filoviruses. We deployed this assay to 15 laboratories of a pandemic preparedness and response consortium, called DURABLE, and report its performance. We demonstrate that diagnostic tests, including an initial external quality assessment (EQA), can be developed and implemented in under two weeks.

2. Material and methods

2.1. Assay

The assay is a hemi-nested PCR targeting the RNA-dependent RNA polymerase gene of filoviruses (Fig. 1, Table S1). It yields a 480 bp (base pair) product in the first round of PCR and 472 bp in the second, with tagged primers for sequencing. An overview of the recommended and used kits is given in Table S2. The assay was previously used by us to detect Bombali virus in a *Mops condylurus* bat [6]. Our primer design targets genomic regions similar to those of He et al., who performed filovirus screening in bats [7].

Primers were synthesized in one batch (Integrated DNA Technologies). Performance of this batch was initially tested using cell culture-derived viral RNA (kindly provided by Felix Drexler, [8]) of MarV and four EboV species: Zaire, Reston, Sudan and Taï Forest. To test specificity, water and extracted RNA/DNA from 40 leftover human plasma samples – from individuals aged 2 to 79 years who had previously tested positive for adenovirus, cytomegalovirus, Epstein-Barr virus, human herpesvirus type 6, and/or BK virus (as single or mixed infections)-were used.

2.2. Positive controls and external quality assessment panel

In vitro transcript (IVT) RNAs of EboV, MarV, and Mengla virus (MeglV) were used as control material (each with 10^5 cps/µL). IVTs were quantified using the Qubit RNA HS Kit (Invitrogen). Due to the short fragment size (<500 bp) and their non-infectious nature, these materials could be sent to all laboratories without major restrictions. Additionally, an EQA panel of blinded samples was generated to assess test performance. The three IVTs were diluted in RNase-free water, yielding six RNA-positive samples at 10^4 and 10^3 cps/µL. Two negative samples (water) were also included in the EQA panel.

2.3. Data analysis

Each laboratory was assigned a specific identifier (L1–15). Images were plotted using GraphPad Prism Version: 9. Probit analysis was performed using R (version: 4.3.0) and the *investr* package (version: 1.4.2).

3. Results

3.1. Assay design and validation

Degenerate bases were used at specific positions of the PCR primers to allow generic detection of filoviruses (Fig. 1). None of the negative samples showed a visible band on the agarose gel, whereas all positive controls showed a band of the expected amplicon size (Figs. S1 and S2). For single-center validation at Charité, three serially diluted IVTs were tested, and probit regression analyses performed. The 95 % lower LOD were 12.7 cps/µL for EboV, 12.6 cps/µL for MarV, and 3.3 cps/µL for MeglV (Fig. 2A).

We deployed primers and protocol together with control material and EQA samples and received results from 15 laboratories from 12 European countries (Fig. S3). Eight of the 15 laboratories used the recommended PCR kits (Table S2).

Laboratories were asked to serially dilute positive controls and test them using the provided protocol. Two laboratories detected all controls at 1 cp/µL and one other lab detected MeglV at this level. The positive controls at 1000 cps/µL were detected by 73.3 % (EboV, 11/15), 73.3 % (MarV, 11/15) and 86.7 % (MeglV, 13/15) of all participants (Fig. S4).

To determine the lower LOD across the laboratories, probit regression analyses were used. Analysis excluded two labs (L9, L12) due to implausible data. The 95 % LOD across remaining labs was 5309 cps/µL (EboV), 10,273 cps/µL (MarV), and 2145 cps/µL (MeglV) (Fig. 2B).

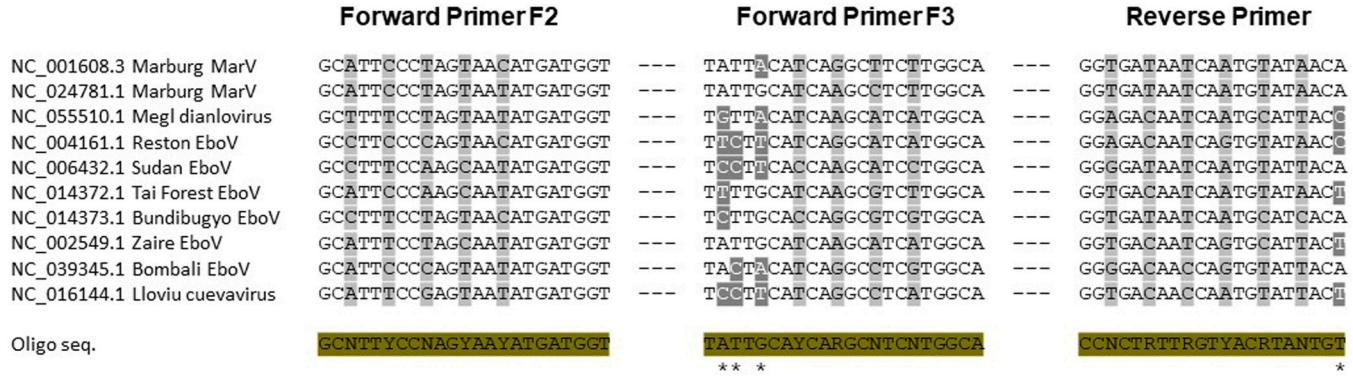

Fig. 1. Alignment of different filovirus species and primer binding sites. MarV, Marburg virus; EboV, Ebola virus; MeglV, Mengla virus; Oligo, oligonucleotide; seq, sequence. Potential mismatches in oligonucleotides are indicated by asterisks. N = any base, R = A/G, Y = C/T.

3.2. EQA

All 15 laboratories correctly detected the two negative EQA samples and the EboV and MarV samples containing 10^4 cps/µL (Table 1). Eleven laboratories identified all EQA samples correctly (73.3 %; 11/15). Two (13.3 %; 2/15) laboratories identified seven samples correctly, and two (13.3 %; 2/15) laboratories identified six samples correctly.

In total, labs made 84/90 (93.3 %) correct positive identifications and 30/30 (100 %) correct negative ones. False negatives were mainly at 10^3 cps/µL (Table 1).

Thirteen out of 15 laboratories (86.7 %) sequenced the PCR products. Of these, 10 used Sanger and 3 used Oxford Nanopore (ONT) sequencing. Two of the laboratories using ONT correctly identified all samples. Nine (69.2 %) laboratories correctly sequenced all positive samples; two sequenced five and one sequenced four. One laboratory had sequencing quality issues.

Table 1
EQA panel composition and performance of laboratories.

			Correct molecular result		Correct virus result	
Sample ID	*Virus*	*Copies/µL*	*%*	*No./total*	*%*	*No./total*
1001	-	-	*100*	*15/15*	-	-
1002	*EboV*	*10,000*	*100*	*15/15*	*90.9*	*12/13*
1003	*MarV*	*10,000*	*100*	*15/15*	*90.9*	*12/13*
1004	*MeglV*	*10,000*	*93.3*	*14/15*	*81.8*	*11/13*
1005	*MeglV*	*1000*	*93.3*	*14/15*	*81.8*	*11/13*
1006	*MarV*	*1000*	*86.6*	*13/15*	*81.8*	*11/13*
1007	*EboV*	*1000*	*86.6*	*13/15*	*81.8*	*11/13*
1008	-	-	*100*	*15/15*	-	-

EboV, Ebola virus; MarV, Marburg virus; MeglV, Mengla virus.

4. Discussion

Here we report the successful validation and distribution of a conventional hemi-nested PCR assay for the detection of filoviruses to 15 laboratories in a European pandemic preparedness consortium. The PCR

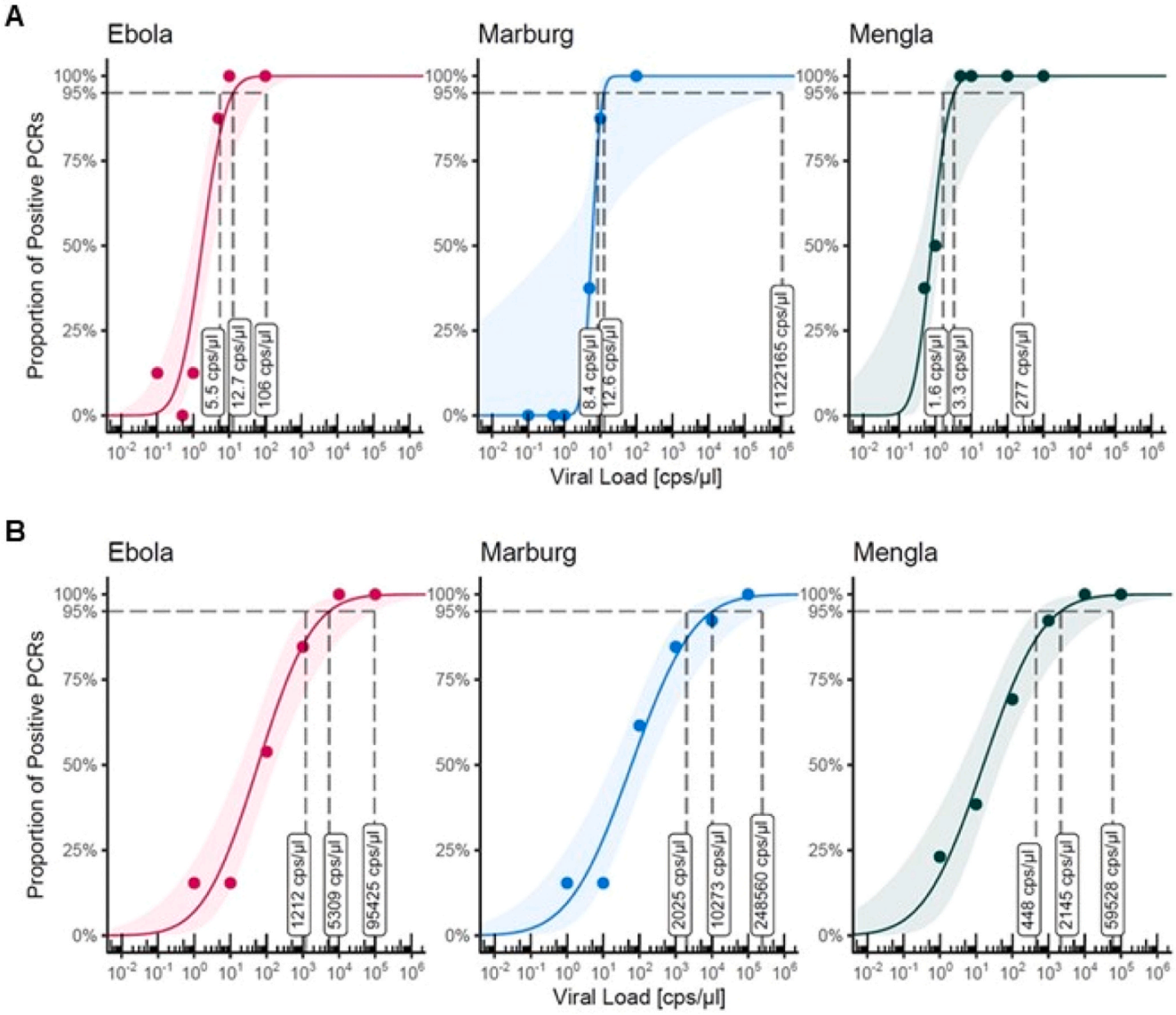

Fig. 2. Probit regression analysis of IVTs. Probability of detection (y-axis) is plotted against RNA copies per µL (x-axis) for three positive controls. Dots: observed fraction of positive results; solid line: predicted proportion of positive results at a given control input concentration; coloured area: 95 % confidence interval for the prediction. from (A) single-center validation. The 95 % LOD were 12.7 cps/µL for EboV, 12.6 cps/µL for MarV, and 3.3 cps/µL for MeglV. (B) The 95 % LOD after multicenter validation were 5309 cps/µL for EboV, 10,272 cps/µL for MarV, and 2145 cps/µL for MeglV.

assay effectively detects several filoviruses and showed an analytical sensitivity of 13 cps/μL in a single-center validation. Multicenter evaluation of the assay was done using *in vitro* transcripts, as working with actual field samples would have required BSL-4 containment, which was not feasible.

The multicenter validation showed variability in assay performance, in particular an increase in the 95 % LOD. Identifying the exact sources of variation is challenging, as variability may result from differences in PCR kits, enzymes [9], and staff experience, especially given the use of the nowadays relatively uncommon two-round PCR method. Primer synthesis can be excluded as all labs used primers from the same batch. However, handling errors remain possible [10]. This also applies to the handling and storage of IVTs, which can affect stability. The observed variability highlights differences in standards and kit availability across countries and therewith reflects the diversity of real-world laboratory conditions. While this is a limitation of the study, it also serves to identify both the strengths and weaknesses of current protocols and highlights the need for greater standardization.

73.3 % of the laboratories correctly identified all EQA samples and no false positive samples were reported. As expected, false negative results were reported mainly in the lower concentration samples (10^3 cps/μL). Sequencing of filovirus-positive samples was performed by 86.7 % (13/15) of participating labs, with only 69.2 % reporting correct results. This underperformance should be addressed by dry-lab EQAs similar to the pilot EQA of SARS-CoV-2 whole-genome sequencing conducted in early 2022, which identified missing data, bioinformatics decisions, and data interpretation as the main causes of discrepancies [11].

In conclusion, we successfully implemented an assay across multiple laboratories in Europe, achieving deployment and initial EQA within two weeks. Establishing efficient distribution networks and laboratory readiness is crucial for maximizing diagnostic capacity for priority pathogens.

While the EQA identified performance issues, it cannot fully mitigate variability in a multi-laboratory setting. A key challenge is the availability and comparability of PCR reagents and cyclers. Our experience highlights the importance of training and knowledge sharing to improve diagnostic accuracy. Standardized sourcing of reagents and comprehensive training programs will further improve overall assay reliability.

CRediT authorship contribution statement

V. Pinho dos Reis: Writing – review & editing, Validation. **A.J. Jääskeläinen:** Writing – review & editing, Validation. **P. Maes:** Writing – review & editing, Validation. **R. Molenkamp:** Writing – review & editing, Validation. **L. Barzon:** Writing – review & editing, Validation. **L.D. Presser:** Writing – review & editing, Validation. **J. Šmahelová:** Writing – review & editing, Validation. **V.M. Corman:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **A. Milewska:** Writing – review & editing, Validation. **C. Drosten:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **S. Pappa:** Writing – review & editing, Validation. **C. Baronti:** Writing – review & editing, Validation. **K.C. Heimsch:** Writing – original draft, Visualization, Validation, Project administration, Methodology, Formal analysis. **R. Cordeiro:** Writing – review & editing, Validation. **I. Tabain:** Writing – review & editing, Validation. **T.D. Best:** Writing – review & editing, Visualization, Software. **K. Huik:** Writing – review & editing, Validation. **T. Bleicker:** Validation, Methodology. **G. Marsili:** Writing – review & editing, Validation.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Victor Corman reports financial support was provided by European Union. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jcv.2025.105808.

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