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Abstract

Background

During the five decades since their discovery, filoviruses of four species have caused human hemorrhagic fever outbreaks: Marburg (MARV) marburgvirus, and Zaire (EBOV), Sudan (SUDV) and Bundybugyo (BDBV) ebolaviruses. The largest, devastating EBOV epidemic in West Africa in 2014-16, has been followed by outbreaks of MARV in Uganda, 2017, and EBOV in Democratic Republic of Congo, 2018, emphasizing the need to develop preparedness to diagnose all filoviruses.

Objectives

The aim of this study was to optimize a new filovirus RT-qPCR to detect all filoviruses, define its limits of detection (LOD) and perform a field evaluation with outbreak samples.

Study design

A pan-filovirus RT-qPCR targeting the L gene was developed and evaluated within the EbolaMoDRAD (Ebola virus: modern approaches for developing bedside rapid diagnostics) project. Specificity and sensitivity were determined and the effect of inactivation and PCR reagents (liquid and lyophilized format) were tested.

Results

The LODs for the lyophilized pan-filovirus L-RT-qPCR assay were 9.4 copies per PCR reaction for EBOV, 9.9 for MARV, 1151 for SUDV, 65 for BDBV and 289 for Taï Forest virus. The test was set at the Pasteur Institute, Dakar, Senegal, and 83 Ebola patient samples, with viral load ranging from 5 to 5 million copies of EBOV per reaction, were screened. The results for the patient samples were in 100% concordance with the reference EBOV-specific assay.

Discussion

Overall, the assay showed good sensitivity and specificity, covered all filoviruses known to be human pathogens, performed well both in lyophilized and liquid-phase formats and with EBOV outbreak clinical samples.

Keywords

Ebola, Marburg, Sudan, Bundibugyo, pan-filo

Background (2577 words)

Members of marburgvirus and ebolavirus genera in the family *Filoviridae* cause highly contagious illnesses with high mortality rate. There are five established species of ebolavirus: Zaire (EBOV), Bundibugyo (BDBV), Sudan (SUDV), Taï Forest (TAFV) and Reston (RESTV) viruses. All five can cause human infections, the first three have caused Ebola virus disease (EVD) outbreaks, TAFV has been associated with only one human case, whereas RESTV has been associated only with asymptomatic human seroconversions. Members of the *Marburgvirus* genus, consisting of the Marburg marburgvirus (MARV) and the Ravn virus (RAVV), also cause severe hemorrhagic fever in humans (Nyakarahuka et al. 2017). The third genus, *Cuevavirus*, is represented by the species Lloviu cuevavirus (LLOV), which hasn't yet been associated with human infections (Negredo et al. 2011). Filoviruses are thought to be zoonotic, and bats are considered the likely reservoirs of these viruses (Olival et al., 2014). The Egyptian fruit bat, *Rousettus aegypticus*, has been identified as the host for MARV (Towner et al., 2009) while the very recently described Bombali ebolavirus (BOMV) and LLOV genome sequences were discovered in samples of insectivorous bats in Africa and Europe, respectively (Negredo et al., 2011; Goldstein et al., 2018). While the exact host of ebolaviruses has not been confirmed yet, genetic and serological evidence of ebolavirus infections have been detected in a few species of fruit bats and most recently insectivorous bats (Leroy et al., 2005; Biek et al., 2006; Swanepoel et al. 2007; Laing et al., 2018; Goldstein et al., 2018). Advances in virus discovery techniques have also yielded detection of novel marburg- and cuevaviruses in bats in China (He et al., 2015; Yang et al., 2017) thus expanding the known range of filoviruses.

The largest Ebola outbreak to date took place in 2014-2016 in both rural and urban areas of Guinea, Sierra Leone and Liberia in West Africa. It was caused by the EBOV, with more than 28 000 reported cases, including more than 11 000 deaths (World Health Organization, WHO; http://www.who.int/csr/disease/ebola/en/). This epidemic highlighted the need for rapid detection of EBOV for disease containment. In response, novel diagnostic tools have been developed for rapid and safe identification of EBOV (reviewed in Clark et al., 2018). The development of efficacious ring vaccination and new treatment modalities further requires efficient diagnostics. Most recently, the WHO reported a new epidemic of EBOV in the Democratic Republic of the Congo (DRC) in May 2018, and an unrelated outbreak some 2500km away in DRC in July 2018 calling for swift response yet again.

Between the EBOV outbreaks, a smaller outbreak of MARV occurred in Uganda in October, 2017, emphasizing the need to develop preparedness to diagnose all filoviruses. Whereas many protocols have been developed recently for detection of EBOV, the detection methods covering the whole range of filoviruses still rely largely on the protocol by Panning et al., 2007. The discoveries of yet novel filoviruses (Goldstein et al. 2018) further underline the need to update and improve the preparedness for rapid and sensitive detection of filoviruses.

Nucleic acid testing is the gold standard for filovirus diagnostics due to high viral loads that become detectable in just a few days after infection (Schurtleff et al. 2015). Such diagnostic tools need to be set up both at the site of the epidemic, and at sites of potential importation. The EBOV outbreak in West Africa activated a broad laboratory response e.g. in Europe (Reusken et al., 2018), with modern molecular diagnostics vastly available for detection of EBOV. For example, freeze-dried PCR reagents would ease the transport and use of the assays in harsh field conditions at the site of epidemic.

Critical steps in nucleic acid testing are biosafe sample preparation and transport. Different protocols for the inactivation of ebolaviruses have been investigated, such as Qiagen AVL buffer and the MagNa Pure Lysis buffer (MPLB; Roche Life Science, Espoo, Finland). The protocol for sampling directly into MPLB has been suggested (Rosenstierne et al. 2016) and this would enable biosafe transport, which is challenging nowadays. Two widely used inactivators are Triton™-X 100, which interferes with lipid membranes, and lysis buffers containing guanidinium thiocyanate that lyse cells and inhibit nuclease activities. Neither of these can alone inactivate ebolaviruses, but parallel use of these two [e.g. Triton™-X 100 together with AVL buffer from Viral RNA mini kit (Qiagen) or MPLB (Roche)] have been shown to fully inactivate ebolaviruses (Rosenstierne et al. 2016; Burton et al. 2017; van Kampen et al. 2017). WHO has recommended the parallel usage of two different inactivation reagents.

Objectives

Here we aimed to develop and evaluate a broad-range pan-filovirus detection method allowing early identification of the causative agent of a filovirus outbreak. We also evaluated the freeze-dried and liquid formats, and possible effects that sample inactivation methods may have on the sensitivity of the assay. Finally, the method was put to test with a large panel of EVD outbreak patient samples.

Study design

Pan-filo L-RT-qPCR

Two different PCR reagents; Superscript® III Platinum® One-step qRT-PCR System (Invitrogen, Carlsbad, CA, USA; later referred as the Invitrogen assay) and lyophilized one-step RT-qPCR reagents (Thermo Fisher Scientific, Massachusetts, USA; later referred as the Thermo-lyophilized assay) were used with the same primers, probes, and viral RNA panels.

A pan-filo L-RT-qPCR targeting the L gene was developed and evaluated. For the assay, two separate reactions were carried out for each sample in the same run.

In the first reaction, 160nM FAM-labelled Filo1 and Filo2 probes (adapted from Jääskeläinen et al. 2015; targeting ZEBOVs) in addition to ZEBOV/MARV reverse primers [480nM of 5'-AATGCATCCAATTAAAAACATTC-3' (Jääskeläinen et al. 2015), 240nM of 5'-AATGCATCCARTCRAATAAATTY-3'] and 240nM ZEBOV/MARV forward primers [5'-AACTGATTTAGAGAAATACAATCTTGC-3' (Jääskeläinen et al.2015), 5'-CTGATCTTGAGAAATACAACCTCGC-3', 5'-ACTGATYTAGAGAAATACAAYCTYGC-3'], and 160nM VIC-labelled Filo3 and Filo4 probes [VIC- TTT ACA CGR CAT TTC ATA GAC T-MGBNFQ and VIC- ACT GTA ATC GAT GTT ATG GT-MGBNFQ; mainly targeting MARVs] were used with final concentration of 2mM $MgSO_4$.

In the second reaction, a final concentration of 240nM of BDBV-RV primer [5'-AATGCATCCAATTGAATAAATTT-3'], 240nM SUDV-RV [5'-CATCCAATCAAAGACATTGC-3'], 320nM FILO-FW [5'-ACMGACCTRGARAAATAYAACYTGGC-3'] in addition to 160nM FAM-labelled Filo5-probe [FAM- ATG AGT TTA CAG CTC CAT T-MGBNFQ; mainly BDBVs] and 160nM VIC-labelled Filo6 probe [VIC- TCA TCA AAT ATT GCA ACC AA-MGBNFQ; mainly targeting SUDVs] were used.

The Invitrogen assay in the Mx3005P qPCR System (Agilent Technologies Finland Oy, Espoo, Finland) was initially used to optimize the concentrations of the primers and probes. Both Invitrogen and Thermo-lyophilized assays were further tested by screening of different filoviruses, and to verify the specificity, and to test the PCR reagent ability to tolerate the inhibition effect of Triton™-X 100 (Sigma-Aldrich, Espoo, Finland), MPLB (Roche), and AVL lysis buffer (Qiagen, Hilden, Germany). Finally, the limits of detection (LODs) were determined for the Thermo-lyophilized assay.

The RT-qPCR running protocol for Invitrogen-liquid assay was adapted from the EBOV assay described in Jääskeläinen et al. (2015) and 7µl of template was used, and the protocol for Thermo-lyophilized assay 3.6µl of template was used and the run was carried out as follows: reverse transcription for 10 min at 45ºC, PCR initial activation step for 10 min at 95ºC followed by 50 cycles of denaturation for 15s at 95ºC, and annealing and extension for 70s at 60ºC.

Viral controls

Quantified *in vitro* RNAs were produced using constructs for EBOV and MARV L genes (described in Jääskeläinen et al. 2015), in addition to synthetized L-gene constructs of SUDV and BDBV ebolaviruses (GeneArt™ Plasmid Construction Service, Thermo Fischer Scientific). RNAs from inactivated whole virus controls of EBOV/Guinea C05 and EBOV/Mayinga, MARV/Angola, RAVV, SUDV/Boniface, BDBV/E76, TAFV/Ivory Coast and RESTV were obtained from Public Health England (PHE, Porton Down, Salisbury, UK). These viruses were cultivated in Vero E6 cells, inactivated, and RNA extracted using QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) according to manufacturer's instruction.

Specificity and inhibition tests

For assessing specificity, EDTA-blood samples from 45 individuals that were sent for routine human herpesvirus 6 nucleic acid testing (later referred as HU-samples) in Helsinki University Hospital (HUSLAB, Helsinki, Finland; anonymous samples, research permit TYH2017257), were extracted using MagNa Pure 96 automated system and nucleic acid kits (MPLB lysis; Roche Life Science, Espoo, Finland), and tested using the Invitrogen and Thermo-lyophilized assays. In addition, for testing other viral hemorrhagic fever agents, the RNAs from inactivated whole virus controls of Lassa virus (strain Liberia; LASV), Dengueviruses 1-4 (DENV1-4), Yellow fever virus (strain 17D; YFV), Rift Valley fever virus (strain RKI; RVFV) and Crimean-Congo hemorrhagic virus (strain Hoti; CCHFV) were extracted using QIAamp Viral RNA Mini kit (Qiagen) (Table 1).

For Triton™-X 100 (Sigma-Aldrich, Espoo, Finland) inhibition tests, excess amount of Triton™-X 100 was added to EDTA-blood samples in final volume of 10% in order to test any PCR inhibition effect due to the reagent in both Invitrogen and Thermo-lyophilized assays. Triton-treated (10 min at room temperature) EDTA-blood samples were extracted using AVL and QIAamp Viral RNA Mini kit (Qiagen). In addition, EDTA-blood samples without extra Triton treatment were extracted using MPLB and MagNA Pure extraction system (Roche). Different amounts of EBOV, MARV, SUDV or BDBV RNAs were spiked in the extracted samples, and both Thermo-lyophilized and Invitrogen assays were carried out (Figure 1).

Sensitivity

Sensitivity of the pan-filo L-RT-qPCR was tested using the Thermo-lyophilized assay and serial dilutions of quantified RNA transcripts (Qubit, Thermo Fisher Scientific) of EBOV, MARV, SUDV and BDBV L-gene, and TAFV (PHE, quantified). Five parallel reactions and Probit Regression (SPSS, IBM) were used to determine LODs. The Invitrogen assay was tested

in parallel with the WHO-approved RealStar® Filovirus RT-PCR Kit (Altona Diagnostics GmbH, Hamburg, Germany) using ZEBOV strain Guinea (range 5-1000 genome copies/PCR reaction; PHE), SUDV L-gene RNA (range 7-1.5E6 genome copies/PCR reaction) and BDBV L-gene RNA (range 13-1.25E6 genome copies/PCR reaction) (Table 1). In addition, whole virus controls (Table 1, PHE) were tested to screen different filovirus targets.

Screening of Ebola patient samples

Samples were collected in Guinea between December 2014 and May 2015 as part of the Institute Pasteur de Dakar (IPD) diagnostics activities of suspected EVD cases (under an emergency response mandate from the government of Guinea and WHO, ref 0235/14/GUI/CPC; all patients agreed to be tested for Ebola virus infection and leftover samples to be used for further investigations). Suspect Ebola patients were defined as any person with recent or past sudden onset of fever and having been in contact with a suspected, probable or confirmed case of EVD, or any person with sudden onset of fever and at least three of the following symptoms: headaches, anorexia/loss of appetite, lethargy, myalgia, arthralgia, breathing difficulties, or any person with inexplicable bleeding. Eighty-three serum samples from acute cases were extracted using QIAamp Viral RNA Mini kit (Qiagen) according to manufacturer´s instructions in IPD, Senegal. These were all EBOV nucleic acid positive using the reference EBOV NP-RT-qPCR (Weidmann et al. 2004; using modified forward primer of 5'-ATGATGGA**R**GCTACGGCG-3' and probe 5'-CA**R**AGTTACTCGGAAAACGGCAT) with viral loads ranging from 5 RNA copies to 5.5 million RNA copies per reaction. In IPD, the pan-filo L-RT-qPCR and EBOV NP-RT-qPCR were carried out in parallel using QuantiTect® Probe RT-PCR kit (Qiagen) and 5µl of template for performance comparison.

Results

Both Invitrogen and Thermo-lyophilized pan-filo L-RT-qPCR assays tested negative for HU-samples (*N*=45), as well as for LASV, DENV1-4, YFV, RVFV and CCHFV, indicating analytical specificity of 100% (95CI: 94.8-100%; Table 1). In addition, both assays detected EBOV/Guinea C05, EBOV/Mayinga, MARV/Angola, RAVV, SUDV/Boniface, BDBV/E76, TAFV/Ivory Coast and RESTV (Table 1).

The LODs (SPSS, Probit, 95CI) for Thermo-lyophilized assay were 9.9 copies/PCR reaction for MARV, 9.4 for EBOV, 65 for BDBV, 1151 for SUDV and 289 for TAFV (PHE).

The Invitrogen pan-filo L-RT-qPCR assay was as good as the RealStar® Filovirus Screen RT-PCR Kit for EBOV, however, for BDBV and SUDV samples there were minor differences (Table 1).

Triton™-X 100 (Sigma-Aldrich), AVL (Qiagen) or MPLB (Roche) did not interfere the Invitrogen or Thermo-lyophilized one-step assays (Figure 1).

All of the patient samples tested (83) from IPD (Senegal) were positive for EBOV nucleic acids using our pan-filo L-RT-qPCR assay and EBOV NP-RT-qPCR (Figure 2) indicating 100% analytical sensitivity. The LOD for EBOV NP-RT-qPCR was 4 copies/PCR reaction (tested in University of Helsinki; SPSS, Probit, 95CI).

Discussion

Filoviruses were first isolated more than 50 years ago, and outbreaks with high mortality have subsequently been caused by four viruses, MARV (9 times) SUDV (5 times), BDBV (twice) and EBOV (14 times) (WHO). For containing and restricting the epidemics, including establishing control measures and therapeutics, accessible, rapid and reliable diagnostic tests

188 are essential. IgM and IgG detection assays are used to confirm resolved disease or to
189 diagnostically monitor samples of cases beyond the diagnostic window for molecular detection.
190 While rapid antigen detection tests with varying sensitivity and specificity have been developed
191 for EBOV detection [Walker et al., 2015; Broadhurst et al. 2015], nucleic acid detection
192 remains the cornerstone of diagnostics. It is a challenge to find simple protocols, particularly
193 primer and probe sequences applicable to all pathogenic filoviruses, yet tests covering all
194 filoviruses would be essential in early identification of outbreaks as well as occasional cases in
195 endemic regions or travelers. Adding such a test and its evaluation is what we report here.
196 However, in future, the lyophilized protocol would benefit from lyophilized primers and probes
197 in mastermixes making the protocol more suitable for field conditions.

198

199 After the West African EBOV epidemic, a variety of methods, inactivation protocols and
200 handling procedures have been studied (Rosenstierne et al. 2016; Burton et al. 2017; van
201 Kampen et al. 2017). Most of the detection methods, however, have been based on the detection
202 of only EBOV nucleic acids, and this limitation can cost time for diagnosis of other filoviruses.
203 At the moment there is only one commercial pan-filovirus kit (RealStar® Filovirus Screen RT-
204 PCR Kit) approved by WHO that also detects MARV, others still aim to detect EBOV antigen
205 or nucleic acids (WHO; http://www.who.int/medicines/ebola-
206 treatment/emp_ebola_diagnostics/en/). These WHO-approved tests include Liferiver™ Ebola
207 Virus (EBOV) Real Time RT-PCR kit (Shanghai ZJ BioTech Co., Ltd) which can be used for
208 detection EBOV, SUDV, TAFV, BDBV, and Xpert® Ebola Test (Cepheid AB, Sweden),
209 FilmArray™ Biothreat-E (BioFire Defence LLC, USA) which both only detect EBOV.

210 The 2-well pan-filo L-RT-qPCR assay described here detected all the tested strains of MARV,
211 EBOV, BDBV, SUDV, TAFV, as well as RESTV. In addition, we were able to validate the

pan-filo L-RT-qPCR assay with clinical samples from the West-African EBOV outbreak with excellent performance. Overall, the assay achieved better performance for EBOV and MARV than rest of the tested targets, but was still at the same level as WHO-approved RealStar® Filovirus Screen RT-PCR kit. With this in mind, it´s recommended to test several samples from patient suspected for VHF to avoid false negative results at the early onset of disease.

The specificity was 100%, and the lyophilized or liquid assays were not affected by common chemical inactivation reagents, *i.e.* MPLB, AVL or Triton™-X 100. These results are in line with Rosenstierne et al. (2016) who tested MPLB and AVL buffers.

Based on the sequences, the pan-filo L-RT-qPCR primers and probes are not a perfect match for BOMV (MF319185; forward primer 4, reverse 2, and VIC-probe 3 mismatches) or other bat-related filoviruses in GenBank (National Center for Biotechnology Information, USA). However, when screening Kenyan bat samples, with the pan-filo L-RT-qPCR we detected one sample positive for filovirus. The bat-related filovirus was later sequenced and identified as BOMV (Forbes et al., Emerging Infectious Diseases 2019, in press).

We conclude that the developed assays, both lyophilized and liquid phase, can be used effectively to screen samples from patients suspected for any known filoviral hemorrhagic fever, and both marburg and ebolaviruses can be detected.

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Conflict of Interest

There are no conflicts of interest.

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Figure 1a and b. Comparing the performance of the pan-filo L-RT-qPCR using Thermo-lyophilized and Invitrogen-liquid assays for potential inhibitory effects of different reagents and inactivation methods [Fig 1a.,Triton™-X 100 treatment (Triton) or Fig 1b. Magna Pure Lysis buffer (MPLB)].

Fig.1a

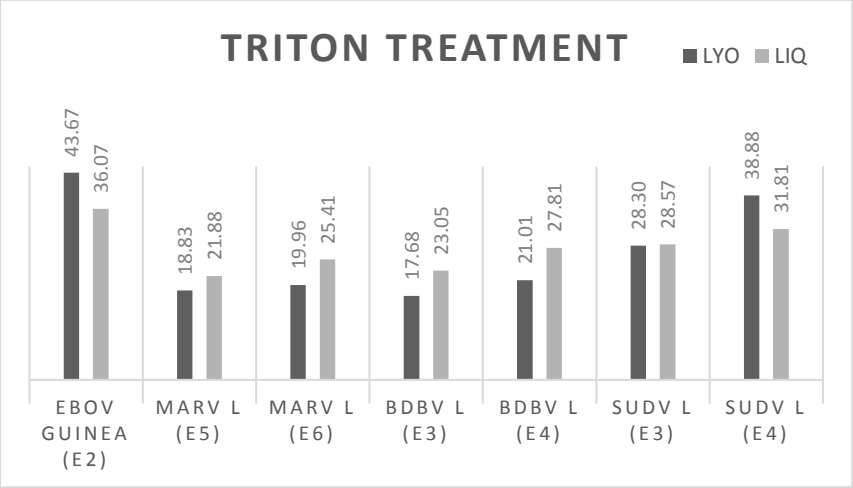
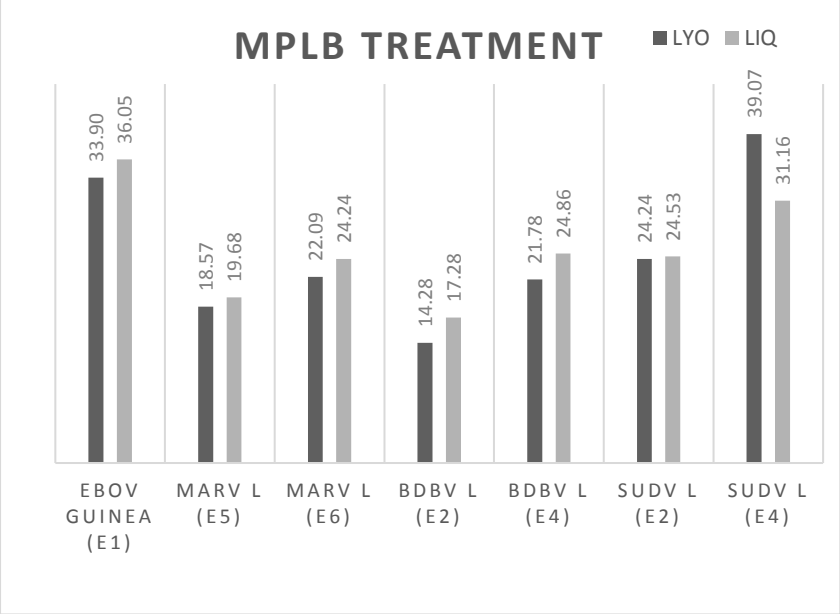


Fig. 1b



Ex, 10^{-x} ($E1=10^{-1}$, $E2=10^{-2}$…) dilution, single replicate; MARV L, L gene construct of Marburg virus; ZEBOV Guinea, RNA extract of Zaire ebolavirus; BDBV L, L gene construct of Bundibugyo virus; SUDV L, L gene construct of Sudan ebolavirus; negat, negative.

Triton: EDTA-blood Triton™-X 100 treated and extracted using Qiagen kit, spiked with filoviral RNA

MPLB: EDTA-blood, Magna Pure LC extracted, using Magna Pure Lysis buffer, spiked with filoviral RNA

Over Ct 40 results are not considered real positive until confirmed by another test if used in diagnosis of filoviral disease in clinical settings. Here Ct-values are listed due to the comparison purposes.

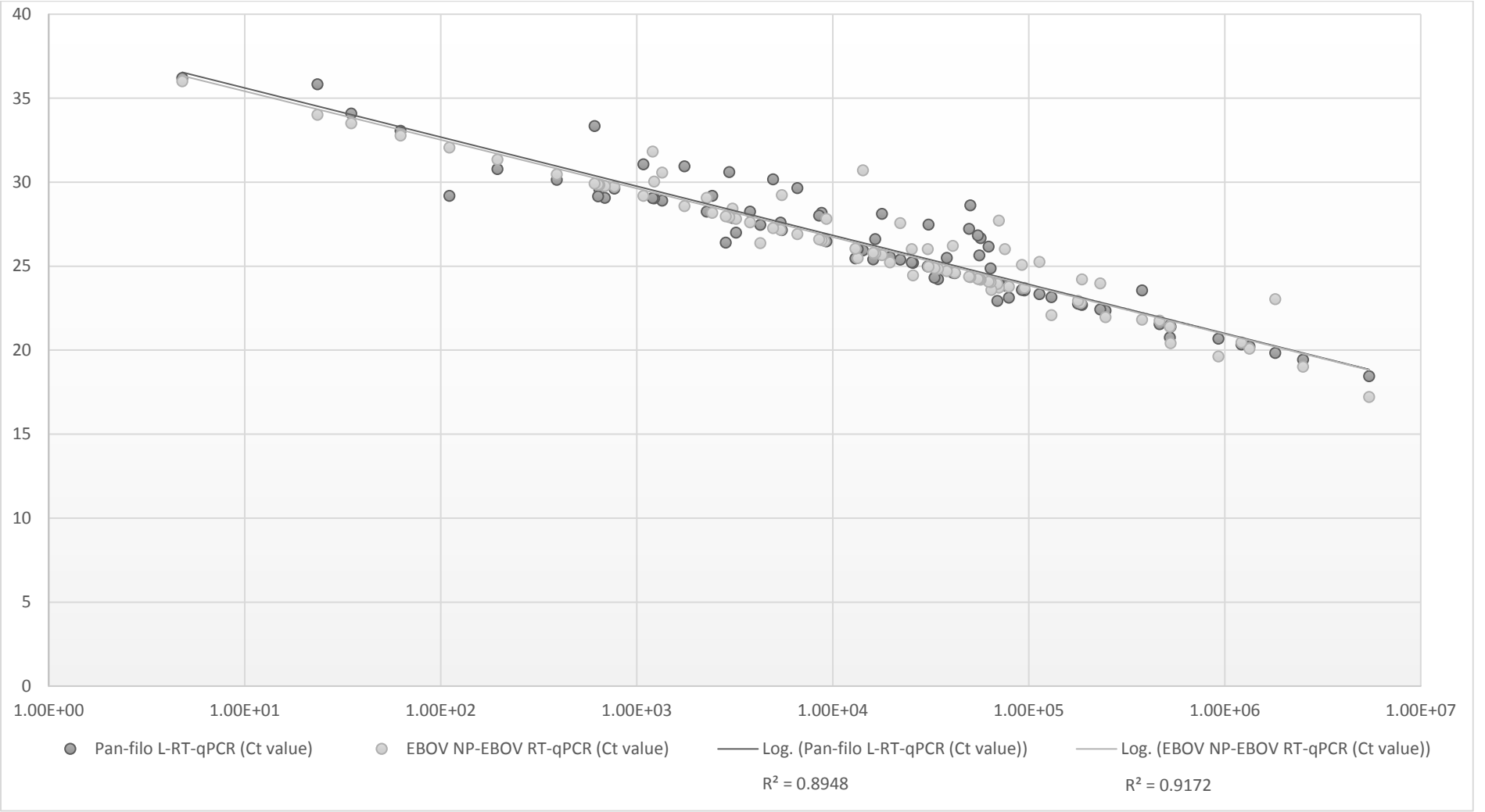


Figure 2. Parallel results of pan-filo L-Rt-qPCR (this study) and EBOV NP-RT-qPCR (Weidmann et al. 2004) assays. The RNA copy numbers (5 copies to 5.5 million copies) of EVD patient samples are presented in the X-axis (logarithmic scale, $\log_{10}$) and Ct values in the Y-axis. R^2, R-squared.

Panel/sample material	Microbial agent	No of samples/tests, neg/pos		Pan-filo L-RT-qPCR assays: Results from Liquid and Lyophilized platforms compared			
		neg for FILO RNA	pos for FILO RNA	neg for FILO RNA		pos for FILO RNA	
				Liq (Invitrogen)	Lyo (Thermo)	Liq (Invitrogen)	Lyo (Thermo)
NEG: Whole blood[1]	Negative sample panel	45	-	45/45	45/45	0/45	0/45
	Total	**45**	**-**	**45/45**	**45/45**	**0/45**	**0/45**
NEG: Viruses[2]	YFV (strains 17D)	3	-	3/3	3/3	0/3	0/3
	DENV1 (RKI)	3	-	3/3	3/3	0/3	0/3
	DENV2 (RKI)	3	-	3/3	3/3	0/3	0/3
	DENV3 (RKI)	3	-	3/3	3/3	0/3	0/3
	DENV4 (RKI)	3	-	3/3	3/3	0/3	0/3
	LASV (strain Liberia, RKI)	3	-	3/3	3/3	0/3	0/3
	RVFV (strain RKI, RKI)	3	-	3/3	3/3	0/3	0/3
	CCHFV (strain Hoti, RKI)	3	-	3/3	3/3	0/3	0/3
	Total	**24**	**-**	**24/24**	**24/24**	**0/24**	**0/24**
All FILO negatives		**69/69 (100%; 95CI 94.8-100%)**					
POS: Viruses/Viral RNAs	ZEBOV (strain Mayinga, PHE)	-	3	0/3	0/3	3/3	3/3
	MARV (strain Angola)	-	2	0/2	0/2	2/2	2/2
	MARV (strain Ravn)	-	2	0/2	0/2	2/2	2/2
	SUDV strain Boniface	-	2	0/2	0/2	2/2	2/2
	BDBV strain E76	-	2	0/2	0/2	2/2	2/2
	Taï Forest virus strain Ivory Coast*	-	2	0/2	0/2	2/2	2/2
	Reston virus	-	2	0/2	0/2	2/2	2/2
	MARV L-gene RNA (HU)*	-	15	0/15	0/15	15/15	15/15

	ZEBOV L-gene construct RNA (HU)	-	15	0/15	0/15	15/15	15/15
	Total	**-**	**45**	**0/45**	**0/45**	**45/45**	**45/45**
POS: 38 viral controls tested using RealStar® Filovirus RT-PCR Kit [3] and <u>Invitrogen assay</u>	**Virus**	**Reference test: RealStar® Filovirus 5 parallel rxns, mean Ct (±SD)**				**Liq, Invitrogen 5 parallel rxns, mean Ct (±SD)**	
	ZEBOV (strain Guinea, PHE)*; 100 copies/rxn	5/5, Ct 30.3 (±0.31)				5/5, Ct 35.6 (±0.66)	
	50 copies/rxn	5/5, Ct 32.3, (±2.7)				4/5, Ct 36.2 (±0.9)	
	25 copies/rxn	1/5, Ct 34.3 (ND)				1/5, Ct 36.1 (ND)	
	Total ZEBOV	**11/15**				**10/15**	
	SUDV L-gene (GeneArt), 1500 copies/rxn	5/5, Ct 31.3 (±3.5)				5/5, Ct 38.3 (±1.3)	
	150 copies/rxn	2/5, Ct 33.7 (±3.8)				1/5, Ct 38.8 (ND)	
	15 copies/rxn	0/5				0/5	
	Total SUDV	**7/15**				**6/15**	
	BDBV L-gene (GeneArt)**, 1250; 625; 125 copies/rxn	1250: Ct 31.4 (ND); 625: Ct 32.0 (ND); 125:Ct 35.4 (ND)				1250: Ct 33.4 (ND), 625: Ct 36.5 (ND), 125: Ct 36.3 (ND)	
	62.5 copies/rxn	1/5, Ct 30 (ND)				4/5, Ct 38.1 (±1.4)	
	Total BDBV	**4/8**				**7/8**	
	Altogether	**22/38**				**23/38**	

Table 1. Sample panels, materials and viruses used for validation of both liquid and lyophilized assay formats of the pan-filo L-RT-qPCR, and the results of the validation.

POS, positive; NEG, negative; FILO, filoviruses; rxn, reaction; ND, not determined; ZEBOV, Zaire ebolavirus; MARV, Marburg virus; BDBV, Bundibugyo virus; SUDV, Sudan ebolavirus; HU, University of Helsinki; PHE, Public Health England; RKI, Robert Koch Institute; DENV,

denguevirus; LASV, Lassa virus; YFV, yellow fever virus; CCHFV, Crimean-Congo Hemorrhagic virus; RVFV, Rift-Valley Fever virus.

** Only one reaction was carried out.

[1]EDTA-Blood samples (Helsinki University Hospital, HUSLAB, Finland)

[2]RNA extractions from inactivated virus cultivations kindly provided by Prof. Niedrig (Robert Koch Institute, Germany).

[3] RealStar® Filovirus Screen RT-PCR Kit (altona Diagnostics GmbH, Hamburg, Germany). Only liquid phase Invitrogen pan-filo L RT-qPCR assay was carried out parallel with RealStar® Filovirus Screen RT-PCR Kit.