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Comparison of Zaire ebolavirus realtime RT-PCRs targeting the nucleoprotein gene

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Abstract

In last five years, the Africa has faced two outbreaks of Zaire ebolavirus. These outbreaks have been the largest so far, and latest outbreak is still ongoing and affecting the Democratic Republic of the Congo.

We tested in parallel three different Zaire ebolavirus (EBOV) realtime RT-PCRs targeting the nucleoprotein gene (EBOV NP-RT-qPCRs) described by Trombley et al. (2010), Huang et al. (2012) and Weidmann et al. (2004). These assays are used regularly in diagnostic laboratories. The limit of detection (LOD), intra-assay repeatability using different matrixes, sensitivity and specificity were determined. In addition, the primers and probes were aligned with the sequences available in ongoing and past outbreaks in order to check the mismatches.

The specificity of all three EBOV NP-RT-qPCRs were excellent (100%), and LODs were under or 10 copies per PCR reaction. Intra-assay repeatability was good in all assays, however the Ct-values were bit higher using the EDTA-blood based matrix. All of the primers and probes in EBOV NP-RT-qPCR assays have one or more mismatches in the probes and primers when the 2267 Zaire EBOV NP sequences, including strains Ituri from DRC outbreak (year 2018), was aligned. The EBOV strain of Bikoro (year 2018) circulating in DRC was 100% match in Trombley and Weidmann assay, but had one mismatch in Huang assay.

1. Introduction

Some of the members in the family *Filoviridae* can cause highly contagious illnesses with high mortality rates. Of these, Zaire ebolavirus (EBOV) is the most common one, and the largest ebola outbreak so far took place in Guinea, Sierra Leone and Liberia in West Africa during the years 2014-2016. In this outbreak, tens of thousands of ebolavirus disease (EVD) cases were reported including approximately 11 000 deaths (World Health Organization, WHO; http://www.who.int/csr/disease/ebola/en/). Shortly after this large outbreak, a new outbreak begun in July 2018 in Democratic Republic of the Congo (DRC), Africa. So far, this outbreak is the second largest outbreak with total of 3392 EVD cases and 2235 deaths (numbers as of 7th of January, 2020; WHO and Ministry of Health, DRC). McMullan et al. (2019) suggested that at least two EBOV strains, Bikoro and Ituri, have crossed the species barrier and are circulating in DRC. This outbreak is still ongoing, especially in the Ituri and North Kivu provinces, DRC.

Many protocols aim for detection of EBOVs, but few comparisons of these methods have been published (Panning et al. 2007; Jääskeläinen et al. 2019; Weidmann et al. 2004; Trombley et al 2010; Huang et al. 2012; Reusken et al 2018). As nucleic acid testing is the gold standard for filovirus diagnostics, comparison of different published primers, protocols and commercial kits are important for decision making and evaluating the impact of negative result of the tests (Schurtleff et al. 2015, Reusken et al 2018).

As a part of the EbolaMoDRAD EU-project, we reviewed and compared three different published EBOV nucleic acid detection protocols and primers targeting the nucleoprotein (NP), which are widely used in, for example, Africa for diagnosis of EVD. To carry out the testing as fluently as possible and to have comparable results, the assays were set up using one commercial PCR reagent kit, and published primers and probes for EBOV NP.

2. Materials and methods

2.1 Zaire ebolavirus NP-RT-qPCRs

Primers and probes, and their final concentrations were adapted from the original publications of three different assays, Weidmann et al. (2004), Trombley et al (2010), and Huang et al. (2012), targeted for EBOV NP. The Weidmann et al. (2004) assay was conducted using the modifications of primers listed in Jääskeläinen et al. (2019). For all of these, Superscript® III Platinum® One-step qRT-PCR System (Invitrogen, Carlsbad, CA, USA; later referred as the Invitrogen assay) and PCR programmed described by Jääskeläinen et al. (2015) were used. Final concentrations of primers were 500nM and 200nM for Weidmann primers and probes, 900nM and 200nM for Trombley, and 400nM and 100nM for Huang, respectively.

2.2 Controls and constructs

Quantified *in vitro* RNAs were produced using a construct for EBOV NP gene (described in Melén et al, 2017) in addition to RNAs from inactivated whole virus controls of EBOV/Guinea C05 and EBOV/Mayinga obtained from Public Health England (PHE, Porton Down, Salisbury, UK), and EBOV/Gabon, Lassa virus (LASV/Liberia), Denguevirus 2 (DENV2), Yellow fever virus (YFV/17D), Rift Valley fever virus (RVFV/RKI) and Crimean-Congo hemorrhagic virus (CCHFV/Hoti) obtained from Robert Koch Institut (Hamburg, Germany; Prof. Niedrig). The RNA from inactivated viruses were extracted using QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) according to manufacturer's instruction. The RNA copy number of EBOV/Guinea was tested using the assay based on glycoprotein (GP) detection described by Trombley et al. (2010) (PHE). EBOV NP RNA transcripts were quantified (Qubit, Thermo Fisher Scientific) and the RNA copy numbers were calculated.

2.3 Limit of detections, specificity and repeatability

Five parallel reactions of different EBOV/Guinea dilutions and Probit Regression (SPSS, IBM; 95 CI) were used to determine limit of detections (LODs). The Weidmann, Trombley and Huang assays were ran in parallel from same EBOV/Guinea or EBOV NP RNA dilutions. For specificity, in addition to LASV, DENV2, YFV, RVFV, CCHFV RNAs, ten EDTA-blood samples (obtained from different Finnish individuals suspected for human herpes virus 6 infection) that were randomly selected from EDTA-blood samples (Helsinki University Hospital, HUSLAB, Helsinki, Finland; anonymous research samples, research permit TYH2017257), were extracted and tested using the different EBOV NP assays. Nucleic acids from the EDTA-blood samples were extracted using MagNa Pure LC system and Total Nucleic Acid isolation kit (Roche Life Science, Espoo, Finland). Intra-assay repeatability in different EBOV/Guinea copy numbers of 5, 10, 25 copies per PCR reaction and EBOV NP construct RNA (95 copies per PCR reaction) was determined using five parallel reactions per run, and percentages of cycle threshold (Ct) coefficient variation (CV) were calculated. The repeatability was tested also using dilutions prepared to both PCR-water and extracted EDTA-blood sample matrix. For the EDTA-blood sample matrix, the EDTA-blood samples from Finnish individuals were extracted using MagNA Pure LC system and Total Nucleic Acid isolation kit (Roche) and then pooled together.

3. Results

The Ct and intra-assay CV% values with different template copy numbers are listed in Table 1. The LODs were 4, 8 and 10 copies per PCR reaction for Weidmann, Trombley and Huang assays, respectively. Negative panel consisting of EDTA-blood samples, in addition to other viral RNAs of LASV, YFV, CCHFV, DENV2, RVF were negative with all tested assays.

4. Discussion

Reusken et al. (2018) have summarized the methods used for diagnosing EVD in Europe. However, in this publication, no comparative data were available. Our goal was to test different RT-qPCRs targeting the EBOV NP. The Weidmann, Trombley and Huang assays are all used for diagnosing of EVD, altogether in seven different countries and laboratories (Reusken et al. 2018).

As shown in this study, the Weidmann, Trombley and Huang assays all detected different EBOVs, such as EBOV/Guinea, Mayinga and Gabon, and *in vitro* transcribed RNA of EBOV NP. The specificity was excellent (100%) in all of the tested assays, and LODs were all under 10 copies per PCR reactions. The Weidmann assay has the lowest LOD value (4 copies per PCR reaction), while Trombley (8 copies per PCR reaction) and Huang assays (10 copies per PCR reaction) seem to have a slightly higher LODs. Weidmann and Huang assays were quite similar when intra-assay repeatability was compared while Trombley assay showed the highest CV% (7%) when 25 copies per PCR reaction of the EBOV/Guinea RNA was used as a dilution in PCR –grade water. The repeatability was similar when EBOV/Guinea was diluted to EDTA-blood based matrix, however, the Ct values and CV% were somewhat higher.

McMullan et al. (2019) showed that the EBOVs from previous outbreaks formed four clades, of which clade 4 comprises of EBOV strains circulated in 2013-2016 in West African epidemic (Guinea, Sierra Leone and Liberia; for example EBOV/Makona, 2015). The EBOV strains causing the ongoing DRC outbreak were clustered in two different clades. These were clade 3 (EBOV/Bikoro, DRC, 2018) and clade 2 (EBOV/Ituri, DRC, 2018). In clade 2, there was also EBOV/Likati causing a small outbreak in DRC in 2017. As the concern of nucleotide mismatches of assay primers and probes to the EBOV strains circulating in DRC, was raised (Mbala-Kingebeni et al. 2019; McMullan et al. 2019), we aligned the Weidmann, Trombley and Huang primers and probes with multiple EBOVs. In figure 1, there are 2267 different EBOV Zaire NP sequences retrieved from the GenBank (NCBI) compared

to primers and probes used in Trombley, Huang and Weidmann assays. In addition, in figure 2, there are EBOV/Makona (2015), EBOV/Mayinga (1976), EBOV/Bikoro (2018), EBOV/Ituri (2018), aligned with primers and probes. The mismatches are shown in figures 1 and 2, and all of the primers and probes had one or more mismatches. With EBOV/Ituri (2018), NP primers used in Trombley assay are 100% identical, although the probe has one mismatch. Huang and Weidmann assays have both single mismatches in primers and probes with EBOV/Ituri (2018). With EBOV/Bikoro, Weidmann assay has 100% match, and Huang assay has one mismatch in probe for all EBOV/Bikoro, Ituri, Mayinga and Makona strains. McMullan et al. (2019) tested Xpert Ebola assay (GeneXpert XVI system, Cepheid, Sunnyvale, CA, USA; targeting EBOV glycoprotein (GP) and NP) and CDC EBOV NP and VP40 real-time RT-qPCR assays, of which all have some mismatches in their primers, but did not detect any decrease in the sensitivity of the assays when DRC strains were tested. Xpert Ebola assay (Cepheid) is widely used in Africa, and Raftery et al (2018) showed the PCR for NP was longer positive than the PCR targeting GP while following up the EVD patients. In our study, the sensitivity of NP assays tested using the quantified EBOV/Guinea was good, it can only be assumed that the sensitivity would be still good for EBOV/Bikoro. However, this should be demonstrated by testing the assays in field conditions and PCR platforms used, for example, in Africa. We know that PCR reagents, conditions and platforms used are affecting the performance of primers and probes, and in addition, mismatches can alter the shape of the amplification curve and the limit of detection of the assay (Wu et al, 2009**;** Klungthong et al, 2010).

5. Conclusion

As part of the EbolaMoDRAD project, the different EBOV NP assay were tasked to be tested in parallel. A comparison of the Weidmann, Trombley and Huang assays was carried out. Overall, all of these assays proved to be sensitive, specific and suitable for diagnosis of EVD, however, the new EBOV/Ituri circulating in DRC should be further tested in order to determine the impact of nucleotide

variation. The sensitivity of the assays for EBOV/Bikoro can be assumed to be as good as for the West African strain of EBOV/Guinea.

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Conflict of Interest

There are no conflict of interests.

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TABLE 1. Comparison of Weidmann et al. (2004), Trombley et al. (2010) and Huang et al. (2012) EBOV NP-RT-qPCR assays.

EBOV NP PCRS			**MEAN CT AND CV%**		
CV%, LOD OR SAMPLES	**Copies/ PCR rxn**	**Info**	**Weidmann et al**	**Trombley et al**	**Huang et al**
CV%, INTRA-ASSAY (5X)	25	EBOV Guinea in EDTA-blood	34.05; 2%	34.10; 1%	35.17; 2%
CV%, INTRA-ASSAY (5X)	10	EBOV Guinea in EDTA-blood	35.61; 4%	35.13; 2%	35.70; 1%
CV%, INTRA-ASSAY (5X)	25	EBOV Guinea in PCR grade water	33.66; 1%	33.51; 7%	34.40; 1%
CV%, INTRA-ASSAY (5X)	10	EBOV Guinea in PCR grade water	34.33; 3%	32.65; 3%	35.95; 4%
CV%, INTRA-ASSAY (5X)	5	EBOV Guinea in PCR grade water	34.95; 4%	33.84; 1%	35.65; 4%
LOD (5X)	LOD	EBOV Guinea in PCR grade water	4	8	10
CV%, INTRA-ASSAY (5X)	95	EBOV NP in-vitro RNA ctrl	32.76; 1%	31.51; 2%	33.15; 1%
NEGATIVE PANEL	10 samples	EDTA-blood samples	All negative	All negative	All negative
EBOV MAYINGA	-	Whole virus RNA	21.60	20.83	22.35
EBOV GUINEA (2014 EPIDEMIC)	Various different quantities	Whole virus RNA	Positive	Positive	Positive
EBOV GABON	-	Whole virus RNA	24.44	25.04	25.51

EBOV, Zaire ebolavirus; NP, nucleoprotein; CV, coefficient variation; LOD, limit of detection; rxn, reaction.

Fig.1 The primer and probe sequences in Trombley et al. (2010), Huang et al. (2012) and Weidmann et al (2004; *modified forward primer and probe listed in Jääskeläinen et al. 2019) were compared to 2267 Zaire Ebola NP sequences retrieved from the GenBank. Sites with rare mismatches are underlined, while more commonly variable sites are both bolded and underlined.

	forward primer	**probe**	**reverse primer**
Trombley assay	TCT GAC ATG GAT TAC CAC AAG ATC	AGG TCT GTC CGT TCA A	GAT TGT TCG GCA AAG AGT CAT CC
Huang assay	GCA GAG CAA GGA CTG AT**T** CA	CAA CAG CTT GGC AAT CAG T**T**G **C**A**G** A	ATT TT**C** CGT TTG ATG CGA AC
Weidmann assay (mod)	ATG ATG GAR G**C**T ACG GCG	CA**R** AGT TA**C** GAA AAC GG CAT	GCA CCA GAT GA**C** TTG GTC CT

Fig.2 Mismatches of primers and probes to carry out the assays described by Trombley et al. (2010), Huang et al. (2012) and Weidmann et al (2004; modified forward primer and probe listed in Jääskeläinen et al. 2019). Two Zaire ebolaviruses from DRC outbreak, the Bikoro (year 2018) and Ituri (year 2018) strains, and two other strains from previous years, Mayinga (year 1976) and West African strain of Makona (year 2015) were used to align the nucleotides of primers and probes.