Establishment of an in-house one-step real-time RT-PCR assay for detection of Zaire ebolavirus

Xuan Su Hoang[1*], Thi Thu Hang Dinh[1*], Van Tong Hoang[1], Huu Tho Ho[1], Tien Sy Bui[2], Van An Nguyen[1], Thai Son Nguyen[1]

[1]Vietnam Military Medical University, Ministry of Defense
[2]108 Military Central Hospital, Ministry of Defense

Received 5 June 2017; accepted 10 October 2017

Abstract:

Ebola virus is a deadly causative agent with a high mortality rate of up to 90%, therefore it has been classified by the Center for Disease Control and Prevention (CDC) as a category A biological agent. The World Health Organization (WHO) recommended using RT-PCR based assays to rapidly detect the virus. In the present study, we established an in-house assay for detection of Zaire ebolavirus via real-time RT-PCR. The nucleotide sequence of the Zaire ebolavirus nucleoprotein (NP) gene was retrieved from the Genbank for designing primer pairs and probes using Primer Express 3.0 software. The RNA positive control was generated by in vitro RNA transcript synthesis. The optimal components in the 20 µl final volume of the real-time RT-PCR assay were 10 µl 2X QuantiTect Probe RT-PCR master mix, 0,6 µM of each primer, 0,1 µM of the probe, 0,2 µl RT mix and 5 µl of RNA template. The thermal cycle conditions were as follows: 50°C for 30 min, 95°C for 15 min, then 45 cycles of 15 s at 94°C, 60s at 60°C. The limit of detection of the assay was 100 copies/reaction and 1414 FFU/ml with the positive RNA panel and sample panel of RNA extracted from cell culture supernatants of cells infected with Zaire ebolavirus 2014/Gueckedou-C05, respectively. The specificity of this assay was 100% when tested with the positive RNA panel of Ebola virus and other haemorrhagic fever viruses. In conclusion, we successfully established an in-house real-time RT-PCR assay for detection of Zaire ebolavirus in Vietnam with a limit of detection of 1414 FFU/ml and specificity of 100%.

Keywords: *ebola virus, real-time RT-PCR, Vietnam, Zaire ebolavirus.*

Classification number: *3.2, 3.5*

Introduction

Ebola virus (EBOV) is a fetal causative agent of severe hemorrhagic fever epidemic with a high mortality rate of up to 90%. The virus was firstly discovered in 1976 when it caused two simultaneous outbreaks in Sudan and Zaire (now Democracy Republic of Congo) [1]. The recent Ebola outbreak in Western Africa was the largest in history with more than 28,602 suspected cases and 11,301 deaths. The cause of this outbreak was then identified as a Makona variant of Zaireebola virus [2]. The WHO declared the outbreak of EBOV disease in West Africa as a "Public health emergency of international concern" and called for a substantial global response in order to control this epidemic [3].

EBOV belongs to the *Filoviridae* family consisting of the five species: Zaire ebolavirus (ZEBOV), Sudan ebolavirus (SEBOV), Reston ebolavirus (REBOV), Bundibugyo ebolavirus (BEBOV) and Tai Forest ebolavirus (TEBOV) [1] EBOV is an enveloped, negative-sense, and single-strand RNA virus with its genome (19 kb in length) encoding for 7 proteins including nucleoprotein (NP), viral protein (VP35), matrix protein (VP40), glycoprotein (GP), replication-transcription protein (VP30), matrix protein (VP24), and RNA dependent RNA polymerase (L). No available vaccines or antiviral drugs exist for prevention and treatment of the EBOV disease. Therefore, early detection of suspected cases is critical for the management, surveillance and control of this deadly epidemic. Real-time RT-PCR assays were used routinely in the laboratory of clinical virology due to high sensitivity, specificity and rapid results, therefore the WHO recommended the use of a real-time RT-PCR assay as the first choice for detection of EBOV in clinical virology laboratories [4]. However, commercial real-time RT-PCR kits approved by the FDA were not available before the arrival of the epidemic in late 2013. Other relevant assays including ELISA, require a Bio safety level 4 (BSL-4) facility for isolation and viral culture [5]. Therefore, a simple, sensitive, and accurate assay based on real-time PCR, which is affordable in countries of limited resources, is essential for

*Corresponding author: Email: hoangxuansu@vmmu.edu.vn

early detection of EBOV in inactivated specimens [6]. This study aims to establish and evaluate a real-time RT-PCR assay for detection of ZEBOV.

Materials and methods

Preparation of ZEBOV RNA positive standard

The 1306 bp nucleotide sequence of a partial NP gene and 3' untranslated region (3'UTR) of recently epidemic ZEBOV strain (GenBank: KJ660348) was chemically synthesized and inserted into the pIDTBlue vector (4 µg) by IDT (USA). This plasmid was linearized by digestion with *Pci*I restriction enzyme for *in vitro* RNA transcription with a Transcript Aid T7 High Yield Transcription Kit (Thermo Scientific), and the synthetic viral RNA transcripts were purified using a GeneJET RNA Purification Kit (Thermo Scientific) according to the manufacturer's instructions. The RNA level was measured by a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific) and then converted to the number of copies per µl. The RNA transcript was stored at -80°C for further use.

RNA extraction

RNA samples were extracted from 140 µl of clinical samples collected from patients in recently Ebola stricken Guinea and from cell culture supernatant of cells infected with ZEBOV2014/ Gueckedou-C05 and other haemorrhagic virus species including SEBOV, REBOV, TEBOV and the Marburg virus [Leiden-BNI 2008], and plasma of patients infected with dengue virus, Zika virus and chikungunya virus for assay cross-reactivity and specificity evaluation using QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. All clinical samples were inactivated before doing extraction by using an AVL buffer and absolute ethanol; then samples were incubated at 60°C for 60 minutes under BSL-4 conditions in the department of virology at Bernhard Notch of Tropical Medicine (BNITM), Hamburg, Germany. Extracted RNA samples were prepared at a concentration of 10^6 copies/ml.

One-step real-time RT-PCR assay

A one-step real-time RT-PCR assay was optimized by using QuantiTect Probe RT-PCR Master mix (Qiagen) in a final volume of 20 µl including 5 µl of RNA. Real-time RT-PCR assays were performed using the Rotor-Gene Q Instrument (Qiagen) as well as LigthCycler 2.0, LighCycler 480 II Instrument (Roche) with thermal cycle parameters as follows: 50°C for 30 min, 95°C for 15 min, then 45 cycles of 15 s at 94°C and 60 s at 60°C. Fluorescent signals were recorded during each annealing step of the amplification cycle and a threshold signal was chosen at 0,1 to determine the threshold cycle (Ct) value during the analysis process for the Rotor-Gene Q Instrument and automated mode for Roche Instrument. All experiments were tested in duplicate within or between runs.

A 10-fold serial dilution from 10^6 to 10^0 copies/µl of transcribed RNA and RNA extracted from cell culture supernatant of infected cells with ZEBOV 2014/Gueckedou-C05 ($1.65x10^5$-$1.65x10^0$ FFU) was used to determine the limit of detection (LoD). The LoD was defined as the lowest RNA concentration detected in all runs of the 20 replicates.

Statistical analysis

The regression and the coefficient of variation (CV) of the mean Ct value for each standard concentration within and between individual PCR runs were analyzed by using statistical excel.

Results

ZEBOV RNA positive standard

The transcribed ZEBOV RNA was yielded with a high concentration of 1,400.3 ng/µl (1.44×10^{12} copies/µl) and 2.01 A260/A280 ratio. Moreover, the RNA transcript was determined by specific size 1806 base in gel agarose electrophoresis (Data not shown). Additionally, the quality of RNA transcript was evaluated by using our previously developed one-step RT-PCR assay for EBOV detection. The RT-PCR product of the ZEBOV RNA in a 10^6 copies/µl concentration is a specific and thick band 830 bp in length (RT mix (+)), whereas there is no band for RT mix (-) RT-PCR (Lane 2 and 3, Fig. 1). Positive RT-PCR product was confirmed exactly by direct sequencing (Data not shown).

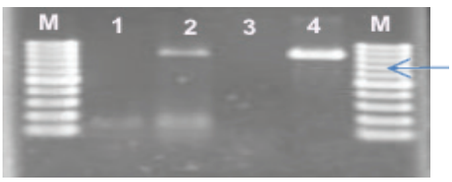

Fig. 1. Evaluation of ZEBOV RNA transcript by one-step RT-PCR assay. M. Marker 100 bp (Thermo Scientific), 1. Negative control; 2. RT-PCR with enzyme RT mix, 3. RT- PCR without enzyme RT mix, 4. Positive control plasmid.

Development and optimization of one-step real-time RT-PCR

Design primer and probe: A nucleotide sequences of the NP gene retrieved from the Genbank database was used for alignment with Clustal W to identify the conserved region for designing a primer and probe. We used Primer Express 3.0 software to design primers in highly conserved regions of the NP gene. The primer and probe sequences were as follows: EBOV-forward: 5'-GACAAATTGCTCGGAATCAC-3'; EBOV-reverse: 5'-ATCTTGTGGTAATCCATGTCAG-3' and probe: 5' FAM - CAGTGAGACTCGGCGTCATCCAGA - TAMRA 3´ that amplified 103 bp in length real-time PCR product (Fig. 2). The primer-probe sequences were checked with a Blast primer tool.

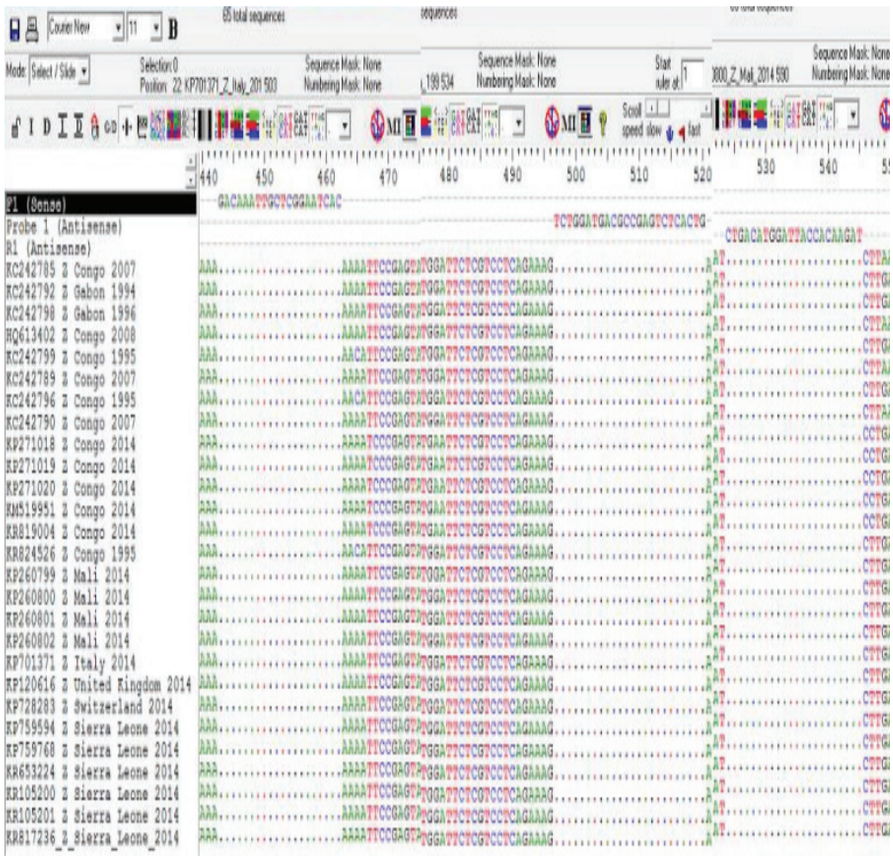

Fig. 2. Nucleotide sequences and sites of primer pairs and probe for a ZEBOV real-time RT-PCR assay.

Table 1. Results of analytical sensitivity.

Concentration	1st Exp Ct	2nd Exp Ct	3rd Exp Ct	Mean Ct	SD	CV
E6	26.39	26.01	27.1	26.5	0.55	0.30
E5	30.16	29.75	32.26	30.7	1.34	1.81
E4	34.67	34.09	36.24	35.0	1.11	1.23
E3	38.84	38.23	40.54	39.2	1.19	1.43
E2	40.41	39.7	43.71	41.2	2.13	4.57
E1	-	-	-	-	-	-

SD: standard deviation, CV: coefficient of variation.

Optimization of the one-step real-time RT-PCR assay: Concentrations of primers and probes were optimized in a final volume of 20 *µl reaction mixture containing* 5 *µl of RNA template to obtain minimal* Ct. Primer concentrations were tested from 0.1 to 0.6 µM and probe concentrations were tested from 0.05 to 0.4 µM. The optimal reaction was obtained at a primer concentration of 0.6 µM (for both primers) and a probe concentration of 0.1 µM.

Limit of detection and specificity of one-step real-time RT-PCR assay

The analytical sensitivity of the real-time RT-PCR assay was evaluated in triplicates on a sample panel ranging from 10^0 to 10^6 copies/µl which was created by serial dilutions of the synthetic viral stock RNAs. The threshold line was chosen at 0.1 during analysis and the data collected were analyzed by linear regression (r^2= 0.99). The results showed that the one-step real-time RT-PCR assays could detect in samples at the concentration of 10^2 copies/reaction (Table 1).

Additionally, the diagnostic sensitivity of the assay was assessed by determination of the LoD, defined as the last dilution at which all replicates were positive. The results have shown the diagnostic sensitivity was 1.65 x 10^1 FFU/reaction, mean 1414 FFU/ml equivalent, indicating a good sensitivity (Fig. 3 and Table 2).

$$\text{Concentration (FFU/ml)} = \frac{1.65 \times 10^1 \text{ FFU/reaction}}{0.14 \text{ ml (V extract sample)} \times \frac{5 \text{ µl (V RNA/reaction)}}{60 \text{ µl (V elute RNA)}}} = 1414 \text{ FFU/ml}$$

The LoD of each test was determined to be the lowest concentration resulting in 95% positive detection of 20 replicates. Furthermore, we also evaluated the sensitivity of the assays on several clinical specimens with different viral loads measured with a Realstar Ebola PCR kit in BNITM. Therefore, the diagnostic sensitivity of the assay was confirmed at the 1.65 x 10^1 FFU/reaction, it was also set as the LoD for the assay. End-point real-time RT-PCR products also showed specific bands with a length of 103 bp on agarose gel (Fig. 4).

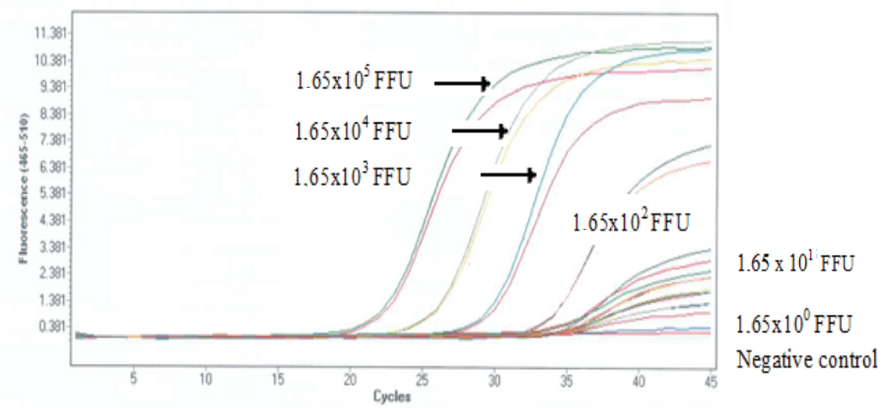

Fig. 3. Concentration dilutions from 1.65 x10^5 to 1.65 x10^0 FFU/reaction.

Table 2. The diagnostic sensitivity and specificity of real-time RT-PCR.

Sample panel ZEBOV RNA	Quantity (FFU/reaction)	Mean Ct value	Replicates	Assay results
Diluted E-1	1.65 x 10^5	22.3	6	100% Positive
Diluted E-2	1.65 x 10^4	26.31	6	100% Positive
Diluted E-3	1.65 x 10^3	29.68	6	100% Positive
Diluted E-4	1.65 x 10^2	33.62	9	100% Positive
Diluted E-5	1.65 x 10^1	34.72	20	100% Positive
Diluted E-6	1.65 x 10^0	-	6	100% Negative
Other viruses				
Sudan EBOV Gulu			3	100% Negative
Reston EBOV			3	100% Negative
Tai Forest EBOV			3	100% Negative
Marburgvirus Leiden			3	100% Negative
Marburgvirus Popp			3	100% Negative
Dengue virus			3	100% Negative
Chikungunya virus S27			3	100% Negative
Zika virus			3	100% Negative

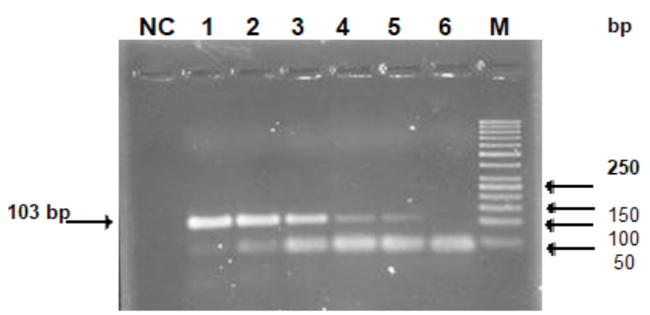

Fig. 4. Representative agarose gel 2% of end-point products of one-step real-time RT-PCR ZEBOV RNA from 1,65 x10^5 to 1.65 x10^0 FFU/reaction. M: marker 50 bp (thermo scientific), NC: negative control; 1-6: 1.65 x10^5 -1.65 x10^0 FFU.

The cross-reactivity and specificity of the assay were tested with RNAs extracted from the supernatant of cell-cultures infected with other EBOV species: SEBOV, REBOV, TEBOV, and Marburg virus [Leiden-BNI 2008], dengue virus, Zika virus and chikungunya virus. There was no cross-reaction of the assay with any of the other EBOV species which were observed. The diagnostic specificity was 100% of all tested samples which were negative for ZEBOV and closely other hemorrhagic fever viruses.

Discussions

EBOV disease is a major public health issue in the world. Among five EBOV species, ZEBOV caused a majority of the outbreaks in Africa with the highest case-mortality rate of up to 90%. After the three week period of incubation, EBOV disease presents with unspecific symptoms and is usually difficult to differentiate from other tropical diseases [7]. Therefore, diagnostic laboratory assays play an important role in confirming or excluding suspected cases [5]. In recent years, several methods for detecting EBOV have been developed for use in clinical virology laboratories, including the use of several assays under Emergency Use Authorization, and others evaluated in a field setting. Due to the fact that EBOV is categorized as a high-hazard pathogen, diagnostic methods including viral culture and isolation require it to be handled in a BSL-4 facility. However, in resource-limited countries, the WHO and CDC have advised that EBOV can be tested in BSL-2 conditions by nucleic acid testing if specimens are inactivated by appropriate methods.

The first real-time PCR assay was developed by Gibb, et al. to detect and differentiate between ZEBOV and SEBOV in patient samples collected during the 2000 Gulu outbreak [8] sensitive, and specific laboratory diagnostic test is needed to confirm outbreaks of Ebola virus infection and to distinguish it from other diseases that can cause similar clinical symptoms. A one-

tube reverse transcription-PCR assay for the identification of Ebola virus subtype Zaire (Ebola Zaire. In addition, the real-time PCR assay measured the viral load in the patients' plasma, which has been shown to be associated with the outcome of the disease. Recent studies have shown that most patients in Western Africa with high viral load associated with a poor prognosis and higher mortality rate [9]. However, there was not a commercial real-time PCR assay approved by the FDA for use upon emergence of the EBOV outbreak in Western Africa, whereas, various laboratory-developed assays have demonstrated significant variability in regards to their sensitivity of detection as well as their reliability [4, 10, 11].

In this study, we established an in-house assay for detection of recent ZEBOV by one-step real-time RT-PCR. Ideally, optimization of assays needs to be performed on EBOV-RNA samples extracted from the stock viral strains, but it is very difficult to acquire this material in Vietnam because there have yet to be any reported cases of EBOV infection. Therefore we used RNA transcribed *in vitro* from a plasmid containing the NP gene of EBOV to generate both the acceptable standards for the optimization of components and appropriate reaction conditions, as well as for the evaluation of the analytical sensitivity of the assay. Furthermore, we validated the established assay with an RNA sample extracted from inactivated cell culture supernatant of infected cells with ZEBOV 2014/Gueckedou-C05 and several clinical samples to determine the LoD and diagnostic sensitivity at the BNITM in Hamburg, Germany. Results showed that the analytical sensitivity of the assay obtained was at a concentration of 10^2 copies/reaction, whereas, specificity was 100% as tested with RNA extracted from other EBOV species and close other hemorrhagic fever viruses. When tested on RNA extracted from the supernatant of infected cells with ZEBOV 2014/Gueckedou-C05 indicated the LoD at a concentration of 1414 FFU/ml and 100% of positive clinical samples. Importantly, we also optimized one-step real-time RT-PCR using a total volume of 20 µl per reaction, making this assay save more reagents. One notable point, the established assay performed on both the Rotor-Gene Q and LightCycler instrument showed a similar performance. Compared with previous studies, the established assay in this study had higher sensitivity and specificity. When comparing this assay to others it can be said to be affordable in cost and to provides accurate results in a short period of time. In addition, the volume of RNA template and related requirements should be considered when comparing this assay to others. Therefore, it is very important to standardize and optimize with more extensive reagents and then validate these assays further in regards to international WHO reference materials.

In conclusion, we developed a highly specific, sensitive assay for the detection of ZEBOV by one-step real-time RT-PCR with the LoD concentration of 1414 FFU/ml, and specificity of 100%. This assay could be used to detect ZEBOV in samples taken from subjects suspected of infection, after returning from travel in infected regions.

ACKNOWLEDGEMENTS

This work was supported by the project entitled "Establishing a realtime RT-PCR assay for detecting Ebola virus", granted by the Ministry of Science and Technology (Vietnam).

The authors would like to acknowledge Toni Rieger, Jonas Schmidt-Chanasit, and Alexandra Bialonski, Bernhard Notch of Tropical Medicine (BNITM), Hamburg, Germany for technical assistance.

REFERENCES

[1] V. Rougeron, H. Feldmann, G. Grard, S. Becker, E.M. Leroy (2015), "Ebola and Marburg haemorrhagic fever", *J. Clin. Virol.*, **64**, pp.111-119.

[2] WHO|Ebola virus disease [Internet], [cited 2016 Dec 25], available from: http://www.who.int/csr/don/archive/disease/ebola/en/.

[3] K.K.W. To, J.F.W. Chan, A.K.L. Tsang, V.C.C. Cheng, K.Y. Yuen (2015), "Ebola virus disease: a highly fatal infectious disease reemerging in West Africa", *Microbes Infect.*, **17(2)**, pp.84-97.

[4] P. Cherpillod, M. Schibler, G. Vieille, S. Cordey, A. Mamin, P. Vetter, et al. (2016), "Ebola virus disease diagnosis by real-time RT-PCR: A comparative study of 11 different procedures", *J. Clin. Virol.*, **77**, pp.9-14.

[5] M.J. Broadhurst, T.J. Brooks, N.R. Pollock (2016), "Diagnosis of Ebola virus Disease: Past, Present, and Future", *Clin. Microbiol. Rev.*, **29(4)**, pp.773-93.

[6] R.J. Shorten, C.S. Brown, M. Jacobs, S. Rattenbury, A.J. Simpson, S. Mepham (2016), "Diagnostics in Ebola virus Disease in Resource-Rich and Resource-Limited Settings", *PLoS Negl. Trop. Dis.*, **10(10)**, p.e0004948.

[7] H. Feldmann, T.W. Geisbert (2011), "Ebola haemorrhagic fever", *Lancet*, **377(9768)**, pp.849-62.

[8] T.R. Gibb, D.A. Norwood, N. Woollen, E.A. Henchal (2001), "Development and evaluation of a fluorogenic 5' nuclease assay to detect and differentiate between Ebola virus subtypes Zaire and Sudan", *J. Clin. Microbiol.*, **39(11)**, pp.4125-4130.

[9] G. Fitzpatrick, F. Vogt, O.B. Moi Gbabai, T. Decroo, M. Keane, H. De Clerck, et al. (2015), "The contribution of Ebola viral load at admission and other patient characteristics to mortality in a médecins Sans Frontières Ebola case management Centre, Kailahun, Sierra Leone, June-October 2014", *J. Infect. Dis.*, **212(11)**, pp.1752-1758.

[10] L. Liu, Y. Sun, B. Kargbo, C. Zhang, H. Feng, H. Lu, et al. (2015), "Detection of Zaire Ebola virus by real-time reverse transcription-polymerase chain reaction, Sierra Leone, 2014", *J. Virol. Methods*, **222**, pp.62-65.

[11] V.G. Dedkov, N.F. Magassouba, M.V. Safonova, A.A. Deviatkin, A.S. Dolgova, O.V. Pyankov, et al. (2016), "Development and evaluation of a real-time RT-PCR assay for the detection of Ebola virus (Zaire) during an Ebola outbreak in Guinea in 2014-2015", *J. Virol. Methods*, **228**, pp.26-30.