Evaluation of a rapid and sensitive RT-qPCR assay for the detection of Ebola Virus

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ABSTRACT

Background: The 2013–2016 Ebola virus disease (EVD) outbreak showed a lack of diagnostic point-of-care methods. Currently, EBOV diagnosis relies on quantitative reverse-transcription-PCR (RT-qPCR), highly specific and sensitive, but requiring skilled personnel and well-equipped laboratories. In field settings, these factors and others, such as samples' time of collection and transportation, determine a prolonged turnaround-time to final results. In outbreak scenarios, a rapid and transportable method could eliminate issues of cohorting suspected and actual EVD patients for lack of diagnostic certainty. The aim of this study was the field evaluation of the new fast, easy-to-use and reliable RT-qPCR assay and platform for EBOV detection, developed in the framework of the EbolaMoDRAD project by CLONIT S.r.l. and STMicroelectronics S.r.l.

Study design: We evaluated its performance during the outbreak and in further studies in the EVD laboratory at the Princess Christian Maternity Hospital (PCMH) in Freetown (Sierra Leone) run by Emergency NGO and the Italian National Institute for Infectious Diseases (INMI). The assay was tested on residual aliquots of clinical specimens from EBOV-positive or −negative patients (n = 116, EVD prevalence 37%).

Results and conclusion: Overall, the test was very easy-to-use and the instrument was robust and reliable in field-settings. The sensitivity of the assay was 100% and the specificity was 98.63% (95%CI: 96.34–100.92%). The positive and negative predictive values were 97.73 (95%CI:94.77–100.68%) and 100%, respectively. The high sensitivity and specificity of this new assay indicate that it is promising for laboratory diagnosis, especially in resource-limited settings.

1. Background

The Zaire ebolavirus (EBOV) species is one of five in the Ebolavirus genus (Ellis et al., 1978; Baize et al., 2014). The 2013–2016 Ebola-outbreak is the largest ever reported, resulting in 28,610 cases and 11,308 deaths, with an average fatality rate of 52%, in the range from 42% in Sierra Leone to 66% in Guinea% (http://www.who.int/csr/don/2014_08_28_ebola/en/http://www.who.int/csr/don/2014_08_28_

ebola/en/). The international community must remain on continuous high alert, as demonstrated by the resurgence of EVD in the north of the Democratic Republic of Congo (DRC) (World Health Organization, 2017).

Since there are no specific medications broadly available to cure Ebola virus disease (EVD), the most important countermeasure is to prevent susceptible people from the infection and restrict transmission (Liu et al., 2017). The prompt and rapid diagnosis is the first step

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towards limiting the propagation of the disease (Nouvellet et al., 2015; Towner et al., 2004).

Since its first use in the field during the 2000 Ebola outbreak in Gabon, quantitative reverse transcription PCR (RT-qPCR) has become the cornerstone for the diagnosis of viral hemorrhagic fevers (VHFs), and RT-qPCR assays have been developed for most of VHF-associated viruses, including EBOV (WHO, 2015).

According to the World Health Organization (WHO), RT-qPCR is the method of choice because rapid antigen tests may give false positive results in the context of low disease prevalence (Woolhouse et al., 2015). RT-qPCRs assays combine high specificity and high sensitivity, allowing an accurate diagnosis in all phases of the disease as this test is able to detect viral RNA in several specimen types, including samples from privileged body districts in convalescent patients. The major flaws addressed to RT-qPCR assays are their requirement for multiple steps, skilled laboratory personnel, substantial training and technical laboratory infrastructure and sophisticated instruments. In field settings, access to accurate, rapid and easy-to-use assays would be a great asset (Colavita et al., 2016) and the outbreak in West Africa urged the development and clinical evaluation of several RT-qPCR tests, which are fit for the field.

2. Objectives

During the outbreak and in the framework of the EbolaMoDRAD project, CLONIT S.r.l. and STMicroelectronics S.r.l., in collaboration with the Italian National Institute for Infectious Diseases "L. Spallanzani" (INMI), developed a prototype of a fast, easy-to-use and reliable RT-qPCR assay for EBOV detection and a miniaturized, portable and robust thermal cycler. Here, we present the first report on the utilization of the RT-qPCR assay and its first evaluation in the field on human specimens.

3. Study design

3.1. Ethic statement

The study was approved by the Sierra Leone Ethics and Scientific Review Committee and Sierra Leone Pharmacy Board (SLESRC 09/06/2015, PBSL 17/08/2015). Based on the national and local guidelines, it was not required to obtain a written informed consent from the patients because the study protocol expected to use residual aliquots of biological samples banked and withdrawal of additional biological samples was not required.

3.2. Sample type

The study was conducted in the Ebola Treatment Center (ETC) in Goderich (Freetown, Sierra Leone) during the outbreak and in the EVD laboratory established in April 2016 at Princess Christian Maternity Hospital (PCMH) in Freetown (Sierra Leone) run by Emergency Onlus NGO (EMR) and INMI (Colavita et al., 2016). Viral nucleic acids extracted from plasma, urine and oral swabs were used. All clinical specimens were collected from EBOV-positive and −negative patients, hospitalized at the EMR ETC from 12 December 2014–21 June 2015; and were tested for EBOV in RT-qPCR with the reference test, performed on a SmartCycler® instrument (Cepheid), during the outbreak. Samples were then anonymized and retrospectively used for the evaluation of the Quanty ZEBOV FAST RT-qPCR on Q3-Plus system.

During the outbreak, inactivation of the clinical samples was performed in the ETC in a Class III Biosafety Cabinet (BSCIII) and RNA extraction was performed with QIAamp® Viral RNA Mini Kit (Qiagen) following manufacturer's instructions. An equal volume of 140 μl was used for the nucleic acid extraction of each sample. During the outbreak, the RT-qPCR assay was evaluated directly on a total number of 29 freshly extracted viral RNAs from plasma and oral swab (27 and 2,

respectively), in parallel with the reference test RealStar® Filovirus Screen RT-qPCR Kit 1.0 (Altona Diagnostics GmbH), which can detect all Ebolaviruses, performed on a SmartCycler® (Cepheid) and has an analytical sensitivity of 1.39 cps/μl (95% CI: 0.69–5.32 cps/μl) (10). Later, residual aliquots (n = 87) of extracted nucleic acids from plasma, oral swab and urine (66, 13 and 8, respectively) were tested. All the residual nucleic acids have been stored at −20 °C under controlled power supply until the second part of the study. To exclude the influence of freeze-thawing cycles on the samples, these were re-tested with the RealStar® Filovirus Screen RT-qPCR Kit 1.0 and no significant difference was evidenced in the Ct values obtained after storage at −20 °C (data not shown).

3.3. Quanty ZEBOV FAST

The kit developed by CLONIT S.r.l., able to detect the viral RNA in one-step reaction, is provided with the ready-to-use mix, which requires a cold-chain for shipping and storage (−20 °C), negative control and three positive controls with different concentrations for the quantification of the sample (synthetic RNA corresponding to NP gene 10^3 cps/μl, 10^4 cps/μl, 10^5 cps/μl). The ready-to-use mix is added in 1:1 proportion to the sample and a total of 20 μl is loaded in each specific cartridge well. The Quanty ZEBOV FAST prototype is specific for the detection of EBOV and does not recognize other Ebola species, as well as an internal control for RT-qPCR quality evaluation. For EBOV, forward and reverse primers were designed to target a conserved region of 65 base pairs in the nucleoprotein (NP) region of EBOV genome (Shabman et al., 2014). In addition, the target region is also found in EBOV messenger RNAs (mRNAs), thus the assay can be used on cellular samples such as blood. Moreover, an internal control (IC) of synthetic RNA, which contributes as a heterologous amplification system to identify possible RT-PCR inhibition and to confirm the integrity of the reagents of the kit, is already included in the ready-to-use mix.

The specificity towards the target and the IC is granted by fluorescent dye labelled probes.

The test result is quantitative and the range of the standard curve is from 10^6 cps/reaction down to 10^4 cps/reaction. The limit of detection (LOD) of the system was determined by Probit Analysis and was found to be 0.374 cps/μl (95% Confidence interval (CI): 0.162 cps/μl–4.9286 cps/μl). Serial dilutions of synthetic RNA, quantified by spectrophotometric analysis and containing the NP region of interest, were performed to complete the Probit analysis.

3.4. Q3-Plus system

The Q3-Plus developed by STMicroelectronics S.r.l. is an integrated, miniaturized (dimensions 14 × 7 x 8.5 cm, weight 300 g) thermal cycler with a system for fluorescence detection and a dedicated software. The platform is composed of three physical components: a computer, an instrument and a disposable lab-on-chip cartridge (Fig. 1). The Q3-Plus system provides an easy-to-use RT-qPCR platform, which can be handled by laboratory technicians with very little extra training. For EBOV RNA amplification, the following thermal cycle profile, of a total duration of 1 h, was used: a single cycle of reverse transcription for 5 min at 50 °C, 1 min at 95 °C for reverse transcriptase inactivation and DNA polymerase activation followed by 45 amplification cycles of 5 s at 95 °C and 30 min 58 °C. The software gives an automated read out on the computer, in a semi-open system: results are automatically given in Ct values at the end of the run and quantification is possible through the standard curve by adding the given concentrations of the standards in the software. The threshold is automatically set by the software and results are easily interpreted. Manual analysis is also possible. The disposable cartridge accommodates six wells. The cartridge wells are made of wax, which solidifies at the end of the run, limiting intra-test contamination risks. The cartridges are stable up to 30 °C. The Q3-Plus system is portable, very robust and does not require any specific
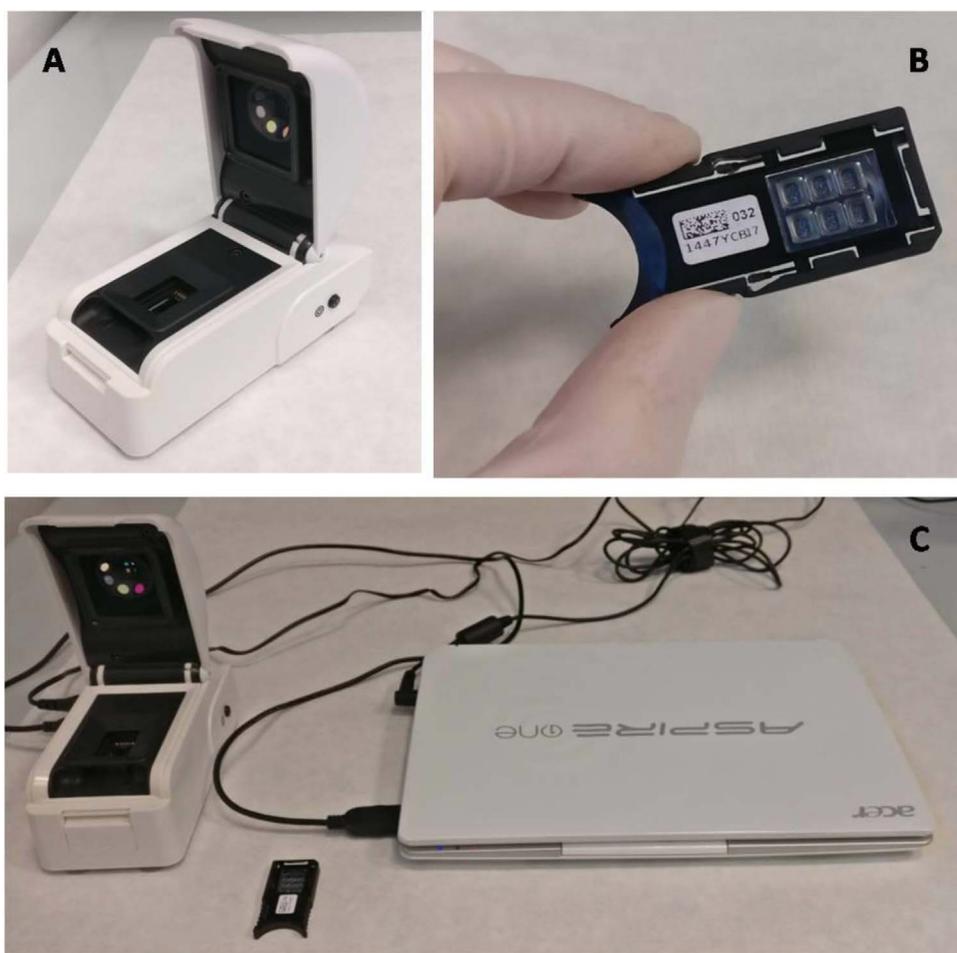
Fig. 1. Q3 Plus Instrument: the system is composed of three physical components: an integrated, miniaturized thermal cycler with a system for fluorescence detection (A); a disposable lab-on-chip cartridge, which accommodates six wells, two for the extracted nucleic acids, one for the negative control, and three for the EBOV RNA standard curve (B); a dedicated computer and software (C).

shrewdness. It is powered with 12 V electricity, it can be run using a car' battery, minimizing the need for complex infrastructures and controlled power supply.

4. Results

4.1. Study group

A total of 116 clinical specimens from EBOV-positive or −negative patients (Table 1) were tested (EVD prevalence of 37%) and 43 samples (32 plasma, 3 oral swabs and 8 urine) resulted EBOV-positive to the reference test RT-QPCR RealStar® Filovirus Screen RT-qPCR Kit 1.0 (Altona Diagnostics) performed on a SmartCycler® (Cepheid). All the EBOV-negative patients analyzed (n = 73) presented with fever > 37.5 °C and either diarrhea or vomiting; two consecutive negative RT-qPCR results were required for their discharge (Bevilacqua et al., 2015).

4.2. Performance of the RT-qPCR assay

Results from the Quanty ZEBOV FAST assay were evaluated comprehensively. On EBOV-positive samples, viral RNA quantitation was evaluated through the standard curve of Quanty ZEBOV FAST mix, included in the kit. Fig. 2 shows comparison of the resulting cps/ml in the different specimen types. Overall, a high sensitivity was observed for most of the EBOV-positive sample (83%) with the Quanty ZEBOV FAST kit; in addition, in our condition, samples type did not seem to influence the overall performance of the Quanty ZEBOV FAST assay.

The diagnostic accuracy was analysed on 116 nucleic acids extracted from plasma, oral swab and urine samples (Table 1). All positive samples (n = 43) that tested positive with the reference test, also tested

Table 1
Perfomance of Quanty ZEBOV FAST assay.

A	RT-qPCR positive [*]	RT-qPCR [*] negative	Total
Quanty ZEBOV Fast positive	43	1	44
Quanty ZEBOV Fast negative	0	72	72
Total	43	73	116

B	Proportion	Percentage (95% CI)
Diagnostic Sensitivity	43/43	100%
Diagnostic Specificity	72/73	98,63% (96,34–100,92)
PPV	43/44	97,73% (94,77–100,68)
NPV	72/72	100%
Diagnostic accuracy	115/116	99,14% (97,31–100,97)

*RT-qPCR (quantitative Real Time PCR) positive to the reference test RealStar® Filovirus Screen RT-PCR Kit 1.0 (Altona Diagnostics GmbH): a Ct value of < 45 was considered positive.
Data are n/N Abbreviations: PPV: positive predictive value; NPV: negative predictive value; CI: 95% confidence interval.

positive with Quanty ZEBOV FAST assay on Q3-Plus system with a diagnostic sensitivity of 100%. The diagnostic specificity was evaluated analysing 73 nucleic acids extracted from plasma samples of patients admitted at the ETC with suspected EBOV infection, who resulted negative to the reference test. Among these samples, 72 were confirmed EBOV-negative and one resulted EBOV-positive with Quanty ZEBOV FAST test (Ct value 32.4). The diagnostic specificity of the test in exam was 98.63% (95% CI: 96.34–100.92%). The overall diagnostic accuracy
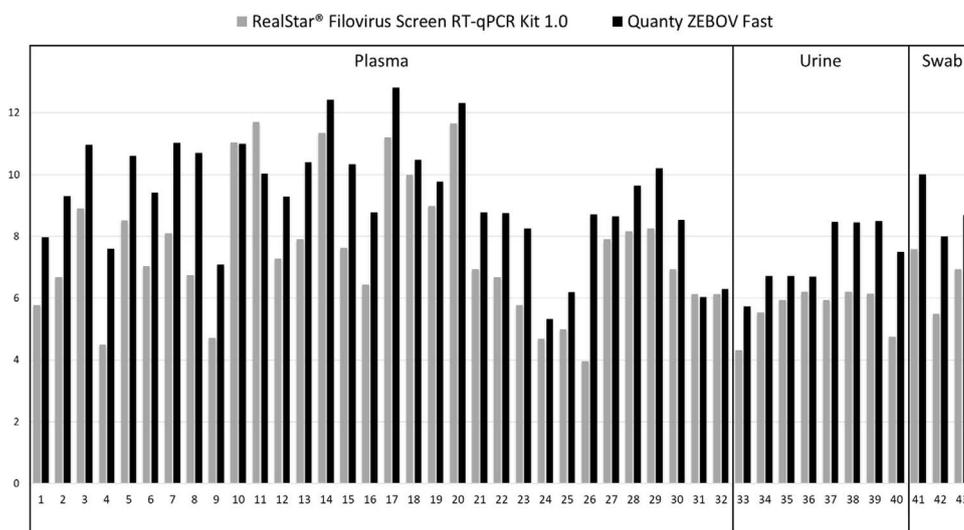
Fig. 2. Quantitation of total EBOV viral RNA in plasma, urine and swab samples. A total of 116 clinical specimens from EBOV-positive or −negative patients (Table 1) were tested (EVD prevalence of 37%, 54 (47%) were male, and the median age was 31 years (interquartile range (IQR): 22–39 years). Plasma, urine and swab clinical specimens were evaluated for the presence of total EBOV viral RNA with Quanty ZEBOV Fast mix and the reference method RealStar® Filovirus Screen RT-PCR Kit 1.0, performed on SmartCycler® (Cepheid). Total viral RNA quantitation was obtained through the standard curve of the respective kit. Quantification of total viral RNA is shown as Log_{10} cps/ml. Symbols are specified in the figures.

was 99.14% (43 + 72 out of 116).

The positive predictive value (PPV) of the RT-qPCR was 97.73% (95% CI: 94.77–100.68%) and the negative predictive value was 100%.

The positive controls and the negative control were evaluated within each run and were reported as EBOV-detected, with the correct cps/ml values, or as EBOV-not detected, respectively. Over the 116 tests, the positive and negative controls gave a 100% reproducibility. Notably, excluding the time required for the nucleic acids extraction, the RT-qPCR time-to-result of the Quanty ZEBOV FAST PCR operated on Q3-Plus system is one hour, almost half the time required for the PCR-reaction performed with RealStar® Filovirus Screen RT-qPCR Kit 1.0, on a SmartCycler® (Cepheid).

5. Discussion

The 2013–2016 Ebola outbreak is the largest ever reported, with an unprecedented number of suspect and confirmed cases. National and world health organizations are ill prepared for countering such infectious disease crisis and evidenced the urgent need for new, more rapid and accurate diagnostic tests for EVD diagnosis, which are currently being developed and implemented. The recent resurgence of EVD in the North of the DRC highlighted the high risk still posed by this disease and the necessity of remaining on alert (World Health Organization, 2017). There are no licensed medications broadly available to cure EVD and the most important countermeasure is to prevent susceptible people from the infection and restrict spreading (Nouvellet et al., 2015; Uyeki et al., 2016). Access to easy and fast diagnosis is essential for patients, their close contacts, and health care workers, and it will eventually lead to a better patient care and management. Recently, different studies evaluated the efficacy of new point of care (POC) tests, either lateral flow devices, antigen detection tests or RT-qPCR, for EVD diagnosis (Boisen et al., 2016; Cnops et al., 2016; Benzine et al., 2016; Broadhurst et al., 2015; Yang et al., 2016). Rapid diagnostic tests such as later flows or rapid antigen detection tests, have the advantage on one side of being rapid, easy-to-use, specific and cheap; but on the other side they are not highly sensitive and are not reliable for samples with low viral load (Broadhurst et al., 2015). On the contrary, the new RT-qPCRs, developed subsequently to the outbreak, resulted highly sensitive and specific, even if some of them were not evaluated under field conditions (Yang et al., 2016).

The Q3-Plus system prototype by STMicroelectronics S.r.l. was developed to address these needs. It is an integrated, miniaturized thermal cycler with a system for fluorescence detection and a dedicated software. The key advantages of this platform are: ease-of-use, it can be handled by laboratory technicians with very little extra training for the

procedures and the results interpretation, extreme portability, and great robustness in constrained settings. In addition, the total duration of the RT-qPCR is of 1 h, a faster assay when compared with the reference test, which has a total duration of 2 h (World Health Organization, 2014). The Q3-Plus instrument is designed to be used with Quanty ZEBOV FAST mix, developed by CLONIT S.r.l., which is ready-to-use master mix and provided with three positive controls for the quantitation of total viral EBOV RNA (both genomic RNAs and mRNAs) in clinical specimens Notably, the platform for now only performs the RT-qPCR reaction and therefore still needs an operational laboratory, which represents a limitation in its applicability. However, the Q3-Plus thermal cycler will be shortly implemented as an integrated system, that also performs nucleic acid extraction directly from clinical specimens, thus easily adapting as a point-of-care diagnostic test in field settings and eliminating the need for skilled personnel and a BSCIII cabinet for samples inactivation.

This study aimed to preliminarily evaluate in outbreak and field settings the Q3-Plus prototype system and the Quanty ZEBOV FAST PCR mix in the framework of the European project Ebola MoDRAD. It was performed on fresh and retrospectively on residual nucleic acids extracted from EBOV positive and negative plasma, oral swab and urine samples, collected from patients hospitalized at the EMR ETC in Goderich (Freetown, Sierra Leone) and stored at −20 °C. The RT-qPCR results were compared with those obtained with the reference test RealStar® Filovirus Screen RT-qPCR Kit 1.0 (World Health Organization, 2014). In our condition, Quanty ZEBOV FAST RT-qPCR, performed on Q3-Plus instrument, showed to have a high sensitivity on the EVD-positive samples tested. However, it must be taken into consideration that the high sensitivity may be due to the higher specificity of the Quanty ZEBOV test, due to its ability to only detect EBOV and not all the Ebolaviruses, as for the reference test. In addition, it has to be considered that, even if it is not likely to have influenced the sensitivity since the results obtained were comparable with those obtained on a manufacturer's recommended instrument, the reference test was performed on SmartCycler®, which is not the recommended platform (Rieger et al., 2016). Furthermore, after the outbreak, some of the samples were tested with RealStar® Filovirus Screen RT-qPCR Kit 1.0 on a Rotor-Gene Q (QIAGEN) instrument, one of the platform recommended by the manufacturer, and no significant difference in Ct values and RNA concentration was highlighted.

The overall diagnostic accuracy was 99.14% (95% IC: 97.3–100.97) for the panel of 116 analyzed samples. Diagnostic sensitivity was 100% and 37% of EVD positive samples had a Ct value ≥ 25, suggesting that the test may be efficient even with those low viral load samples not detected by other assays (Cnops et al., 2016). The diagnostic specificity

was 98.63% (95% IC: 96.34–100.92), thus allowing rapid and accurate identification of the disease and application of the isolation measures, reducing the risk of further transmission. The only EBOV negative sample which resulted positive to the Quanty ZEBOV test had a CT value of 32.4, suggesting a high sensitivity; unfortunately, a second sample of the patient was not available to confirm this result. Limitation of this study is the lack of specificity testing, including cross-reactivity testing against other hemorrhagic fevers and viral pathogens endemic in the outbreak area, which was not carried out in the field, and will be a target for further analysis.

In summary, we evaluated a fast and easy-to-use RT-qPCR test for the rapid diagnosis of EVD. This preliminary study shows its better performance compared to the reference test, indicating that it is promising for laboratory diagnosis in resource-limited settings. Further studies will be needed to fully evaluate the performance of the test, its possibilities and applicability.

Conflict of interest

Acknowledgements

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