Simultaneous detection of Marburg virus and Ebola virus with TaqMan-based multiplex real-time PCR method

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Funding information
Key Scientific and Technological Project of Meizhou People’s Hospital, Grant/Award Number: MPHKSTP-20180101; Guangdong Provincial Key Laboratory of Precision Medicine and Clinical Translational Research of Hakka Population, Grant/Award Number: 2018B030322003; Scientific Research Cultivation Project of Meizhou People’s Hospital, Grant/Award Number: PY-C2020033

Received: 8 January 2021 | Revised: 18 March 2021 | Accepted: 31 March 2021
DOI: 10.1002/jcla.23786

Abstract

Background: Marburg virus (MARV) and Ebola virus (EBOV) are acute infections with high case fatality rates. It is of great significance for epidemic monitoring and prevention and control of infectious diseases by the development of a rapid, specific, and sensitive quantitative PCR method to detect two pathogens simultaneously.

Methods: Primers and TaqMan probes were designed according to highly conserved sequences of these viruses. Sensitivity, specificity, linear range, limit of detection, and the effects of hemolysis and lipid on real-time qPCR were evaluated.

Results: The linearity of the curve allowed quantification of nucleic acid concentrations in range from $10^3$ to $10^9$ copies/ml per reaction (MARV and EBOV). The limit of detection of EBOV was 40 copies/ml, and MARV was 100 copies/ml. It has no cross-reaction with other pathogens such as hepatitis b virus (HBV), hepatitis c virus (HCV), human papillomavirus (HPV), Epstein-Barr virus (EBV), herpes simplex virus (HSV), cytomegalovirus (CMV), and human immunodeficiency virus (HIV). Repeatability analysis of the two viruses showed that their coefficient of variation (CV) was less than 5.0%. The above results indicated that fluorescence quantitative PCR could detect EBOV and MARV sensitively and specifically.

Conclusions: The TaqMan probe-based multiplex fluorescence quantitative PCR assays could detect EBOV and MARV sensitively specifically and simultaneously.

KEYWORDS
Ebola virus, Marburg virus, polymerase chain reaction, simultaneous detection
1 | INTRODUCTION

Filoviruses such as Marburg virus (MARV) and Ebola virus (EBOV) can cause severe hemorrhagic fevers in humans and primates, and as one of the most dangerous pathogens for humans listed by the World Health Organization (WHO). MARV and EBOV are mainly transmitted by direct contact and aerosol. The incubation period is commonly 3–9 days, the elderly can be more than 2 weeks. A variety of body fluids, such as secretions, excretions, contaminants, and environments from patients with hemorrhagic fever and animals infected with MARV or EBOV can infect a variety of immune cells through damaged skin, eyeballs, nasal passages, and oral mucosa. Patients can present with serious bleedings and hemorrhagic shock syndrome. It has high pathogenicity and fatality rate.

MARV was first discovered in Marburg, Germany in 1967 and was named Marburg virus according to the location of this disease. It was the first filovirus found in human. MARV is a member of the filoviruses family, has caused outbreaks in sub-Saharan Africa, and can cause severe disease with a high case fatality rate. Human-to-human transmission may occur in a home or hospital setting.

Although MARV was discovered more than 50 years ago, there is no effective treatment has yet been developed, except the vaccine has a preventive effect. The first outbreak of the EBOV occurred in the Ebola River region in southern Sudan in 1976, patient mortality rates as high as 90%. In the outbreak in West Africa between 2013 and 2016, there were about 28,000 cases were confirmed and 11,000 deaths were reported, indicating the high mortality rate of this disease.

Although the viruses have only been endemic in some African countries, and no large-scale virus-infected patients have been reported in other regions, there is a potential risk of virus transmission owing to countries around the world interact more and more with each other and the movement of people and goods increases. Moreover, MARV and EBOV can be used as a potential biological terrorist weapons or biological agents to use. Therefore, it is necessary to establish a rapid and specific laboratory test method for the early diagnosis and prevention of MARV and EBOV infection. TaqMan-based real-time fluorescence quantitative polymerase chain reaction (PCR) has the characteristics of rapid, sensitive, specific, and high throughput, and has been used in the detection of a variety of viruses.

MARV and EBOV has a negative-strand RNA of approximately 19 kb, respectively. This RNA genome contains seven genes, including nucleoprotein (NP), virion protein (VP) 35, VP40, glycoprotein (GP), VP30, VP24, and the RNA-dependent RNA polymerase L. Each one of these genes encodes a single protein product. We aimed to establish a rapid, sensitive TaqMan-based real-time fluorescence quantitative PCR detection assays according to the genetic sequence of the viruses, to provide technical support for laboratory testing of prevention and control of these severe infectious diseases.

2 | MATERIALS AND METHODS

2.1 | Identification of sequences and design of primers and probes

According to the whole genome sequence of 42 strains of MARV published by GenBank, DNASTAR software was used for multiple sequence alignment (MSA) to screen the highly conserved NP gene of MARV nuclear protein as the target gene. According to the whole genome sequence of EBOV published by GenBank, DNASTAR software was used for multiple sequence alignment to screen out the highly conserved NP gene of EBOV as the target gene.

Primer Premier 6.0 software was used to design specific primers and probes that met multiple response conditions. Multiple primers were designed with the following default settings: primer melting temperature (Tm) set at 60°C approximately. TaqMan probes for EBOV and MARV were labeled at the 5′-end with the reporter molecule: hexachloro-6-carboxy-fluorescein (HEX), pentamethine cyanine (CY5), respectively. And the TaqMan probes were labeled at the corresponding 3′-end with the quenchers: 6-carboxy-tetramethyl-rhodamine (TAMRA), 8-Bromo-7-hydroxyquinoline 2 (BHQ2) (Sangon Biotech Co., Ltd.), respectively. And the size of PCR product of EBOV and MARV were 163 and 180 bp, respectively (Table 1).

| Species      | Primer/probe | Sequence (5′–3′)       | Probe type | Fluorescent reporter dye at the 5′ ends | Quenching group at the 3′ ends | Product size (bp) |
|--------------|--------------|------------------------|------------|----------------------------------------|-------------------------------|-------------------|
| Ebola virus  | EBL-FP       | GAGAAAAGGCTTGGCTTGGAG  | TaqMan     |                                        |                               | 163               |
|              | EBL-RP       | CATGTCACCCCTTGTTGGTA   |            |                                        |                               |                   |
|              | EBL-Prb      | CCAACACGTCTGGCAATACAGTG |       |                                        |                               |                   |
| Marburg virus| MRB-FP       | CACAGTTTGTGGAGGTTGGGT  | TaqMan     |                                        |                               | 180               |
|              | MRB-RP       | ATGCTCAACACACACACTGCA  |            |                                        |                               |                   |
|              | MRB-Prb      | ACTGCCCTCAGTCTCTAATAA  | CY5        |                                        |                               |                   |

Abbreviations: BHQ2, 8-Bromo-7-hydroxyquinoline 2; CY5, pentamethine cyanine; HEX, hexachloro-6-carboxy-fluorescein; TAMRA, 6-carboxy-tetramethyl-rhodamine.
2.2 | Gene synthesis and preparation of standard substance positive template

The highly conserved sequences of MARV NP gene and EBOV NP gene were synthesized artificially as the target genes and cloned into pUC57 vector through gene synthesis. The gene synthesis was completed by Sangon Co., Ltd.

The cloned strain of pUC57 was cultured in E. coli and plasmid pUC57 was extracted. The absorbance (A) of plasmid at wavelengths of 260 and 280 nm was measured by spectrophotometer, and purity was determined according to the ratio of A260/A280. The plasmid concentration was determined and converted to copy number of plasmid according to the following formula. Dissolve a certain amount of plasmid in the blood and calculate the template concentration and copy number in the blood according to the following formula.

\[
\text{copy number (copies/ml)} = \frac{\text{plasmid concentration (g/ml) \times 6.02 \times 10^{23}}}{(\text{total length of plasmid} \times 660)}
\]

or: \(\text{copy number (copies/μl)} = \frac{\text{plasmid concentration (ng/μl) \times 6.02 \times 10^{23} \times 10^{-9}}}{(\text{total length of plasmid} \times 660)}\)

\((6.02 \times 10^{23}) \) was Avogadro’s constant, 660 was the average molecular weight of each base.

Finally, the template was stored at −20°C for reserve.

2.3 | Optimization of fluorescence quantitative PCR reaction system and reaction conditions

TaqMan-based real-time PCR was performed on the Lightcycler 480 fluorescence quantitative PCR system (Roche Diagnostics). First, a single system qualitative reaction was performed for each pathogen. On this basis, the primer concentration, probe concentration, and \(T_m\) of the multiple fluorescence quantitative PCR reaction were optimized to establish the optimal reaction system for simultaneous detection of two viruses.

A master mix reaction was prepared and dispensed in 23 μl aliquots into the PCR reaction tubes. Then 2 μl of template nucleic acid was added to each tube. The final reaction mixture contained 120 nmol/L of each primer and probe for MARV, 200 nmol/L of each primer, and probe for EBOV. The mixture was heated for reverse transcription at 50°C for 10 min, followed by one cycle of denaturation at 95°C for 5 min. PCR amplification was carried out for 45 cycles at 95°C for 15 s and 58°C for 40 s. The fluorescence was read at the end of this second step allowing a continuous monitoring of the amount of template nucleic acid.

2.4 | Verification of method performance

2.4.1 | Preparation of standard curve

The plasmid standards of \(1.0 \times 10^8, 1.0 \times 10^7, 1.0 \times 10^6, 1.0 \times 10^5, 1.0 \times 10^4, 1.0 \times 10^3, 1.0 \times 10^2, \) and \(1.0 \times 10^1\) copies/ml were repeated in batches, and each template was tested three times. The threshold cycle (Ct) values obtained and the accuracy and stability of the detection system were calculated by SPSS 21.0 software.

2.4.2 | Sensitivity test

The plasmid of EBOV and MARV nucleic acid concentrations of \(1.0 \times 10^6, 1.0 \times 10^7, 1.0 \times 10^8, 1.0 \times 10^9, 1.0 \times 10^{10}, 1.0 \times 10^{11}, \) and \(1.0 \times 10^{12}\) copies/ml were prepared in batches and each template was tested three times. Real-time fluorescence quantitative PCR was used for detection and statistical analysis to determine its sensitivity.

2.4.3 | Specificity test

The specificity of the assays was verified by the existence or absence of amplification using MARV template nucleic acid corresponding to EBOV primers and probe or vice versa. In addition, potential cross-reaction was assessed using serum samples of viral nucleic acid from seven viral species (such as hepatitis B virus (HBV), hepatitis C virus (HCV), human papillomavirus (HPV), Epstein-Barr virus (EBV), herpes simplex virus (HSV), cytomegalovirus (CMV), and human immunodeficiency virus 1 (HIV-1)), which are a part of the collection samples of Center for Precision Medicine, Meizhou People’s Hospital.

2.4.4 | The effect of lipid and hemolysis on the detection of the assay

The influence of lipid and hemolysis on the amplification performance of the assay was investigated. The 50 serum samples with heavy, moderate, and mild blood lipid and hemolysis were collected, respectively, each separate serum 2 ml, stored at −20°C. Lipid and hemolysis samples of EBOV and MARV nucleic acid templates with final concentrations of \(2.0 \times 10^6\) copies/ml (I level), \(3.0 \times 10^6\) copies/ml (II level), and \(4.0 \times 10^4\) copies/ml (III level) were prepared with standard plasmid. Real-time fluorescence quantitative PCR was used for detection and statistical analysis to study whether lipid and hemolysis affected the detection results.

3 | RESULTS

3.1 | Preparation of standard curve

A 10-fold series of diluted plasmids were used as templates for amplification, and the corresponding standard curve was established. The standard curve range was \(4.0 \times 10^1-1.0 \times 10^9\) copies/ml. The results showed that the copy number had a good correlation with the corresponding Ct value, and the correlation coefficient \(R^2 > 0.99\),...
indicating that the standard sample was quantified and the correlation between each concentration group was reliable (Figure 1).

3.2 The results of only MARV detection, simultaneous detection, and only EBOV virus detection of two viruses with various mixtures of synthesized two plasmids

The two viruses mixtures of synthesized plasmids with EBOV and MARV nucleic acid templates final concentrations of $5.0 \times 10^8$, $5.0 \times 10^6$, and $5.0 \times 10^4$ copies/ml were prepared, respectively. PCR reaction performances were determined by the existence or absence of amplification when only MARV detection, simultaneous detection, and only EBOV detection, respectively. In the samples with two plasmid templates, if only MARV primers and probes were added into the reaction system, the CY5-labeled MARV probe showed fluorescence signal while the HEX-labeled EBOV probe showed no fluorescence signal, indicating amplification of MARV template, but EBOV was not amplified. Similarly, EBOV has similar results. In the samples with two plasmid templates, if MARV and EBOV were detected at the same time, both Cy5 and HEX-labeled probes showed fluorescence signals, indicating that both MARV and EBOV templates were amplified (Figure 2).

3.3 Determination of linear range and limit of fluorescence quantitative PCR

The sensitivity of the MARV and EBOV TaqMan-based fluorescence quantitative PCR detection assay were evaluated by using the serial dilutions of the synthesized two plasmids. A Ct value was calculated from the amplification plot covering this range of dilutions and the standard curves were drawn. The linearity of the curve allowed quantification of nucleic acid molecules in range from $10^2$ to $10^7$ copies/ml per reaction (both MARV and EBOV). The limit of detection of EBOV and MARV was 40 and 100 copies/ml, respectively.

3.4 Repeatability analysis of fluorescence quantitative PCR

Three concentrations ($1.0 \times 10^6$, $1.0 \times 10^5$, and $1.0 \times 10^4$ copies/ml) of EBOV and MARV plasmids were determined repeatedly ($n = 3$). According to statistical analysis, the final measured values of EBOV were $1.081 \times 10^6$, $1.118 \times 10^5$, and $1.016 \times 10^4$ copies/ml, with coefficients of variation (CV) of 3.03%, 3.72%, and 2.18%, respectively. And the final measured values were $1.219 \times 10^6$, $1.261 \times 10^5$, and $1.204 \times 10^4$ copies/ml for MARV, with CVs of 3.42%, 4.15%, and 4.38%, respectively. Repeatability analysis showed that their CVs were lower than 10%, indicating high repeatability of the method.

**FIGURE 1** Standard curves obtained with serial dilutions of EBOV and MARV in vitro from PCR products. Ct values calculated from results in fluorescence quantitative PCR are plotted against the log of the initial starting quantity of nucleic acid (copies/ml). The results showed that the set copy number had a good correlation with the corresponding Ct value, and the correlation coefficient $R^2 > 0.99$
was less than 5.0%. These indicating that this method has good accuracy and repeatability.

3.5 | Determination of specificity

The specificity of the assays was verified by the existence or absence of amplification using MARV template nucleic acid corresponding to EBOV primers and probe or vice versa. In addition, potential cross-reactions were assessed using viral nucleic acid from seven viral species samples, which are a part of the collected samples of Center for Precision Medicine, Meizhou People’s Hospital. It has no cross-reaction with other pathogens such as hepatitis b virus (HBV), hepatitis c virus (HCV), human papillomavirus (HPV), Epstein-Barr virus (EBV), herpes simplex virus (HSV), cytomegalovirus (CMV), and human immunodeficiency
virus 1 (HIV-1). The results showed no cross-reaction and good specificity of the assay.

### 3.6 The effect of lipid and hemolysis on the performance of the assay

There were significant differences of final measured values in the I level of EBOV ($p = 0.002$) and MARV ($p < 0.001$) affected by different degrees of hemolysis (Table 2). There were significant differences of final measured values in the I level of EBOV ($p = 0.001$) and MARV ($p < 0.001$) affected by different lipid turbidity (Table 3). There were no significant differences of final measured values in the II, III of EBOV and MARV affected by different lipid turbidity and the degree of hemolysis (Tables 2 and 3; Figure 3). Hemolytic specimens can be properly diluted to avoid interference. For lipid blood specimens, it is better to use high-speed centrifugation to extract the clear solution.

### 4 DISCUSSION

Common laboratory detection methods for virus include isolation and culture,25 transmission electron microscopy (TEM),26 antigen capture ELISA,22,23 immunoglobulin IgM and IgG antibody ELISA,24 reverse transcription PCR (RT-PCR),25,26 real-time quantitative PCR (RT-qPCR) detection and immunohistochemical staining.29,30 Isolation and culture of the virus is very sensitive, but it must be performed in a biosafety level 4 laboratory (BSL-4). Transmission electron microscopy (TEM) is a rapid detection technology, which usually requires very advanced equipment and laboratory. Therefore, these two methods are rarely used in laboratory detection. Currently, there are three detection methods commonly used in the process of outbreak: (1) antibody ELISA; (2) antigen-capture ELISA; (3) molecular biological detection technology based on real-time PCR.31

The commonly used real-time fluorescence PCR techniques include TaqMan probe-based real-time quantitative PCR and dye-based real-time quantitative PCR. The advantages of TaqMan probe method lies in its high specificity and low risk of contamination of samples, but the disadvantage is the high cost of probe synthesis. The method of SYBGreen dye is more general but less specific than that of fluorescent probe.32 In recent years, with the development of fluorescence PCR technology, a variety of real-time fluorescence PCR methods for hemorrhagic fever viruses have been established. TaqMan probe-based fluorescence PCR detection method for 28 hemorrhagic fever viruses has been established by Pang et al., 45-150 copies of synthetic viral RNA can be detected.33

Currently, most laboratories generally use PCR as their preferred and fastest diagnostic method for emergency detection and clinical samples.34 Real-time fluorescent quantitative PCR based

**TABLE 2** Effects of different hemolysis on TaqMan probe real-time quantitative PCR detection

| Group | Severe hemolysis | Moderate hemolysis | Mild hemolysis | Normal sample | $p$ Value |
|-------|------------------|--------------------|----------------|---------------|-----------|
| Ebola virus | | | | | |
| I level ($n = 3$) | $9.413 \times 10^7 \pm 2.868 \times 10^7$ | $7.567 \times 10^7 \pm 7.508 \times 10^6$ | $9.413 \times 10^7 \pm 2.868 \times 10^7$ | $1.907 \times 10^8 \pm 2.994 \times 10^7$ | 0.002 |
| II level ($n = 3$) | $2.320 \times 10^4 \pm 1.019 \times 10^4$ | $1.937 \times 10^5 \pm 3.800 \times 10^5$ | $2.153 \times 10^4 \pm 2.739 \times 10^5$ | $3.317 \times 10^6 \pm 2.122 \times 10^5$ | 0.072 |
| III level ($n = 3$) | $3.253 \times 10^4 \pm 6.901 \times 10^3$ | $2.523 \times 10^4 \pm 4.008 \times 10^3$ | $2.569 \times 10^4 \pm 3.991 \times 10^3$ | $3.347 \times 10^4 \pm 5.601 \times 10^3$ | 0.183 |
| Marburg virus | | | | | |
| I level ($n = 3$) | $8.937 \times 10^7 \pm 7.671 \times 10^6$ | $1.283 \times 10^8 \pm 8.733 \times 10^6$ | $1.227 \times 10^8 \pm 1.193 \times 10^7$ | $2.147 \times 10^8 \pm 2.376 \times 10^7$ | <0.001 |
| II level ($n = 3$) | $3.453 \times 10^4 \pm 4.061 \times 10^4$ | $3.437 \times 10^5 \pm 5.463 \times 10^5$ | $2.683 \times 10^4 \pm 3.513 \times 10^5$ | $3.513 \times 10^4 \pm 1.531 \times 10^5$ | 0.092 |
| III level ($n = 3$) | $4.140 \times 10^4 \pm 9.823 \times 10^3$ | $3.850 \times 10^4 \pm 3.300 \times 10^3$ | $2.867 \times 10^4 \pm 1.457 \times 10^3$ | $3.563 \times 10^4 \pm 7.784 \times 10^3$ | 0.179 |

**TABLE 3** Effects of different lipid turbidity on TaqMan probe real-time quantitative PCR detection

| Group | Severe lipid turbidity | Moderate lipid turbidity | Mild lipid turbidity | Normal sample | $p$ Value |
|-------|------------------------|--------------------------|----------------------|---------------|-----------|
| Ebola virus | | | | | |
| I level ($n = 3$) | $9.477 \times 10^7 \pm 2.911 \times 10^7$ | $2.677 \times 10^8 \pm 3.331 \times 10^7$ | $1.937 \times 10^8 \pm 2.994 \times 10^7$ | $1.577 \times 10^8 \pm 2.639 \times 10^7$ | 0.001 |
| II level ($n = 3$) | $2.877 \times 10^6 \pm 8.373 \times 10^5$ | $3.647 \times 10^6 \pm 2.511 \times 10^5$ | $3.473 \times 10^6 \pm 2.139 \times 10^5$ | $3.683 \times 10^6 \pm 1.976 \times 10^5$ | 0.197 |
| III level ($n = 3$) | $4.880 \times 10^4 \pm 8.448 \times 10^3$ | $7.503 \times 10^4 \pm 2.547 \times 10^4$ | $5.717 \times 10^4 \pm 1.339 \times 10^4$ | $4.330 \times 10^4 \pm 3.869 \times 10^3$ | 0.131 |
| Marburg virus | | | | | |
| I level ($n = 3$) | $1.227 \times 10^8 \pm 1.193 \times 10^7$ | $2.737 \times 10^8 \pm 1.365 \times 10^7$ | $2.147 \times 10^8 \pm 2.376 \times 10^7$ | $2.247 \times 10^8 \pm 2.627 \times 10^7$ | <0.001 |
| II level ($n = 3$) | $5.017 \times 10^4 \pm 2.710 \times 10^4$ | $5.103 \times 10^4 \pm 3.086 \times 10^5$ | $4.513 \times 10^4 \pm 1.531 \times 10^5$ | $5.010 \times 10^4 \pm 2.536 \times 10^5$ | 0.075 |
| III level ($n = 3$) | $3.850 \times 10^4 \pm 3.300 \times 10^3$ | $3.563 \times 10^4 \pm 2.363 \times 10^3$ | $3.423 \times 10^4 \pm 3.101 \times 10^3$ | $3.517 \times 10^4 \pm 3.296 \times 10^3$ | 0.404 |
on fluorescent probe can simultaneously detect EBOV and MARV. The single-tube multiplex PCR method adopted in this study can simultaneously detect EBOV and MARV in one reaction tube. The linearity of the curve allowed quantification of nucleic acid molecules in a range from $10^3$ to $10^9$ copies/ml per reaction in the assay. The limit of detection for EBOV and MARV was 40 and 100 copies/ml, respectively. It has no cross-reaction with other pathogens such as HBV, HCV, HPV, EBV, HSV, CMV, and HIV-1. In addition, repeatability analysis of the two viruses showed that their coefficient of variation (CV) was less than 5%, indicating that the method has good repeatability. The above results indicated that fluorescence quantitative PCR could detect EBOV and MARV sensitively and specifically. Multiplex fluorescent quantitative PCR has advantages in the rapid diagnosis of mixed infection. Multiple real-time fluorescent PCR can simultaneously detect multiple target sequences in one reaction, so it has the advantages of large detection flux, fast detection speed, simple operation, and low detection cost. It is superior to the single-virus real-time PCR assay.
Both EBOV and MARV are virulent viruses, we could not test the virus or positive clinical samples to verify the detection assay, which is the biggest shortcoming of this study. In addition, although relatively conservative fragments are selected for amplification and detection in the design of the assay, there is the possibility of mutation of pathogen genes. Although the probability of mutation in the conservative region selected for amplification detection is very small, this possibility cannot be completely avoided theoretically. Moreover, PCR-based detection has shortcomings and cannot produce absolutely reliable results under any circumstances. PCR-based technologies are susceptible to contamination and lead to false-positive results, and the consequences of false-positive and false negative results of qPCR tests can have serious implications for outbreak management, especially in the early stages of disease and the early rehabilitation of survivors.\(^{35,36}\)

At present, the research on these viruses is still relatively few and the depth of research is not enough in China. Although the natural epidemics of these viruses occur only in individual countries and regions, they are likely to become invasive due to countries around the world interact more and more with each other and the movement of people and goods increases. And they have the potential to become a means of bioterrorism or war. Accordingly, we must pay close attention to virulent virus, and the detection method for virulent virus continues to study. In this study, we established a TaqMan probe-based multiplex fluorescence quantitative PCR detection assay to detect EBOV and MARV, to provide technical support for laboratory testing of prevention and control of these severe infectious diseases.

The emergence of complex global public health crises such as climate change and extremes, biodiversity loss, and the global rise of resistant antibiotics has resulted in an unprecedented rise in direct and indirect mortality and morbidity. Public health emergencies occur frequently.\(^{37}\) The risk of mixed infection with multiple pathogens continues to increase. Real-time fluorescence PCR technology is a method that adds fluorescence molecules to the PCR reaction system, monitors the whole PCR process by real-time detection of the accumulation of fluorescent signals. The technology has the characteristics of high sensitivity and specificity, multiple reactions, high degree of automation, no pollution, real-time, and accuracy.\(^{38}\) It provides technical support for rapid diagnosis of mixed infection. It is feasible to develop multiplex real-time assayable a greater number of dangerous viruses based on TaqMan-based fluorescence PCR.

## 5 CONCLUSION

The TaqMan probe-based multiplex fluorescence quantitative PCR assays could detect EBOV and MARV sensitively, specifically, and simultaneously. This study might provide technical support for laboratory testing of prevention and control of these severe infectious diseases. The further validation of this detection assay and developing multiplex real-time PCR assayable a greater number of dangerous viruses will be the focus of our next work.

## ACKNOWLEDGEMENTS

The author would like to thank other colleagues whom were not listed in the authorship of Center for Precision Medicine, Meizhou People’s Hospital (Huangtang Hospital), Meizhou Hospital Affiliated to Sun Yat-sen University for their helpful comments on the manuscript. This study was supported by Key Scientific and Technological Project of Meizhou People’s Hospital (Grant No: MPHKSTP-20180101 to Dr. Zhixiong Zhong), the Guangdong Provincial Key Laboratory of Precision Medicine and Clinical Translation Research of Hakka Population (Grant No: 2018B030322003), and the Scientific Research Cultivation Project of Meizhou People’s Hospital (Grant No: PY-CZ2020033 to Dr. Zhikang Yu).

## CONFLICT OF INTEREST

The authors declare that they have no competing interests.

## AUTHOR CONTRIBUTIONS

Heming Wu and Zhixiong Zhong designed the study. Heming Wu and Zhikang Yu performed the experiments. Qingyan Huang helped to analyze the data. Heming Wu prepared the manuscript. All authors were responsible for critical revisions, and all authors read and approved the final version of this work.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## REFERENCES

1. Feldmann H, Klenk HD. Marburg and Ebola viruses. Adv Virus Res. 1996;47(2):1-52.
2. Emanuel J, Marzi A, Feldmann H. Filoviruses: Ecology, molecular biology, and evolution. Adv Virus Res. 2018;100:189-221.
3. Leffel EK, Reed DS. Marburg and Ebola viruses as aerosol threats. Biosecur Bioterror. 2004;2(3):186-191.
4. Rivera A, Messaoudi I. Molecular mechanisms of Ebola pathogenesis. J Leukoc Biol. 2016;100(5):889-904.
5. Hall RCW, Hall RCW, Chapman MJ. The 1995 Kikwit Ebola outbreak: lessons hospitals and physicians can apply to future viral epidemics. Gen Hosp Psychiatry. 2008;30(5):446-452.
6. Peterson AT, Lash RR, Carroll DS, Johnson KM. Geographic potential for outbreaks of Marburg hemorrhagic fever. Am J Trop Med Hyg. 2006;75(1):9-15.
7. Brauburger K, Hume AJ, Mühlberger E, Olejnik J. Forty-five years of Marburg virus research. Viruses. 2012;4(10):1878-1927.
8. Akpovwa H. Chloroquine could be used for the treatment of filoviral infections and other viral infections that emerge or emerged from viruses requiring an acidic pH for infectivity. Cell Biochem Funct. 2016;34(4):191-196.
9. Suschak JJ, Schmaljohn CS. Vaccines against Ebola virus and Marburg virus: recent advances and promising candidates. Hum Vaccin Immunother. 2019;15(10):2359-2377.
10. Pourrut X, Kumulungui B, Wittmann T, et al. The natural history of Ebola virus in Africa. Microbes Infect. 2005;7(7):1005-1014.
11. Garske T, Cori A, Ariyarajah A, et al. Heterogeneities in the case fatality ratio in the West African Ebola outbreak 2013–2016. *Philos Trans R Soc Lond B Biol Sci*. 2017;372(1721):20160308.

12. Peterson AT, Samy AM. Geographic potential of disease caused by Ebola and Marburg viruses in Africa. *Acta Trop*. 2016;162:114-124.

13. Hartman AL, Towner JS, Nichol ST. Ebola and Marburg hemorrhagic fever. *Clin Lab Med*. 2010;30(1):161-177.

14. Lucht A, Grunow R, Otterbein C, Möller P, Feldmann H, Becker S. Partial validation of a TaqMan real-time quantitative PCR for the detection of ranaviruses. *Dis Aquat Organ*. 2018;128(2):105-116.

15. Shao N, Li F, Nie K, et al. TaqMan Real-time RT-PCR assay for detecting and differentiating Japanese encephalitis virus. *Biomed Environ Sci*. 2018;31(3):208-214.

16. Noorbazargan H, Nadji SA, Mirab Samiee S, Paryan M, Mohammadi-Yeganeh S. Comparison of a new in-house HIV-1 TaqMan real-time PCR and three commercial HIV-1 RNA quantitative assays. *Comp Immunol Microbiol Infect Dis*. 2018;59:1-7.

17. Rojas M, Monsalve DM, Pacheco Y, et al. Ebola virus disease: An emerging and re-emerging viral threat. *J Autoimmun*. 2020;106:102375.

18. Volchkov VE, Volchkova VA, Chepurnov AA, et al. Characterization of the L gene and 5’ trailer region of Ebola virus. *J Gen Virol*. 1999;80(Pt 2):355-362.

19. Schmidt KM, Möhlberger E. Marburg virus reverse genetics systems. *Virology*. 2016;8(6):178.

20. Upadhyay RK, Roy P. Deciphering dynamics of recent spread and outbreak in West Africa: The case of Ebola virus. *Int J Bifurcat Chaos*. 2016;26(9):1630024.

21. Nanni L, Paci M, Brahman S, Hyttinen J. Analysis of virus textures in transmission electron microscopy images. *Stud Health Technol Inform*. 2014;207(21):83-91.

22. Lucht A, Grunow R, Otterbein C, Möller P, Feldmann H, Becker S. Production of monoclonal antibodies and development of an antigen capture ELISA directed against the envelope glycoprotein GP of Ebola virus. *Med Microbiol Immunol*. 2004;193(4):181-187.

23. Miethe P, Gary D, Hlawatsch N, Gad AM. Rapid detection of EBOLA VP40 in microchip immunofiltration assay. *Proc Natl Acad Sci U S A*. 2015;9490:94900H.

24. Chao D, Galula JU, Shen W, Davis BS, Chang GJ. Nonstructural protein 1-specific immunoglobulin M and G antibody capture enzyme-linked immunosorbent assays in diagnosis of flaviviral infections in humans. *J Clin Microbiol*. 2015;53(2):557-566.

25. Rieger T, Kerber R, El Halas H, et al. Evaluation of RealStar reverse transcription–polymerase chain reaction kits for filovirus detection in the laboratory and field. *J Infect Dis*. 2016;214(suppl 3):S243-S249.

26. Kurosaki Y, Takada A, Ebihara H, et al. Rapid and simple detection of Ebola virus by reverse transcription-loop-mediated isothermal amplification. *J Virol Methods*. 2007;141(1):78-83.

27. Wang R, Sheng Z-M, Taubenberger JK. Detection of novel (Swine Origin) H1N1 influenza virus by quantitative real-time reverse transcription-PCR. *J Clin Microbiol*. 2009;47(8):2675-2677.

28. Cnops L, Van den Eede P, Petitt J, et al. Development, evaluation, and integration of a quantitative reverse-transcription polymerase chain reaction diagnostic test for Ebola virus on a molecular diagnostics platform. *J Infect Dis*. 2016;214:5192-5202.

29. Pantin-Jackwood MJ. Immunohistochemical staining of influenza virus in tissues. *Methods Mol Biol*. 2014;1161:51-58.

30. Georgsson G, Houwers DJ, Stefansson K, Plisson PA, Pfuursson G. Immunohistochemical staining of cells in the brain of a patient with acquired immune deficiency syndrome (AIDS) with a monoclonal antibody to visna virus. *Acta Neuropathol*. 1987;73(4):406-408.

31. Rougeron V, Feldmann H, Grard G, Becker S, Leroy EM. Ebola and Marburg haemorrhagic fever. *J Clin Virol*. 2015;64:111-119.

32. Navarro E, Serrano-Heras G, Cano MA, Solera J. Real-time PCR detection chemistry. *Clin Chim Acta*. 2015;439:231-250.

33. Pang Z, Li A, Li J, et al. Comprehensive multiplex one-step real-time TaqMan qRT-PCR assays for detection and quantification of hemorrhagic fever viruses. *PLoS One*. 2014;9:e95635.

34. Mayer FJ, Ratzinger F, Schmidt RLJ, et al. Development of a fully automated high throughput PCR for the detection of SARS-CoV-2: The need for speed. *Virology*. 2020;111(1):964-967.

35. López-Sann Martin M, Cataneo G, Grau A, Valencia JM, Garcia-March JR, Navas JI. Real-Time PCR based test for the early diagnosis of Haplosporidium pinnae affecting fan mussel Pinna nobilis. *PLoS One*. 2019;14(4):e0212028.

36. de Boer P, Rahaoui H, Leer RJ, et al. Real-time PCR detection of *Campylobacter* spp.: A comparison to classic culturing and enrichment. *Food Microbiol*. 2015;51:96-100.

37. Durrheim DN, Gostin LO, Moodley K. When does a major outbreak become a Public Health Emergency of International Concern? *Lancet Infect Dis*. 2020;20(8):887-889.

38. Xie S, Yu H, Wang Q, Cheng Y, Ding T. Two rapid and sensitive methods based on TaqMan qPCR and droplet digital PCR assay for quantitative detection of Bacillus subtilis in rhizosphere. *J Appl Microbiol*. 2020;128(2):518-527.

How to cite this article: Yu Z, Wu H, Huang Q, Zhong Z. Simultaneous detection of Marburg virus and Ebola virus with TaqMan-based multiplex real-time PCR method. *J Clin Lab Anal*. 2021;35:e23786. https://doi.org/10.1002/jcla.23786