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ORIGINAL ARTICLE

Development of a highly sensitive real-time nested RT-PCR assay in a single closed tube for detection of enterovirus 71 in hand, foot, and mouth disease

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Abstract Enterovirus 71 (EV71) is one of the major causative agents of outbreaks of hand, foot, and mouth disease (HFMD). A commercial TaqMan probe-based real-time PCR assay has been widely used for the differential detection of EV71 despite its relatively high cost and failure to detect samples with a low viral load (*Ct* value > 35). In this study, a highly sensitive real-time nested RT-PCR (RTN RT-PCR) assay in a single closed tube for detection of EV71 in HFMD was developed. The sensitivity and specificity of this assay were evaluated using a reference EV71 stock and a panel of controls consisting of coxsackievirus A16 (CVA16) and common respiratory viruses, respectively. The clinical performance of this assay was evaluated and compared with those of a commercial TaqMan probe-based real-time PCR (qRT-PCR) assay and a traditional two-step nested RT-PCR assay. The limit of detection for the RTN RT-PCR assay was 0.01 $TCID_{50}$/ml, with a *Ct* value of 38.3, which was the same as that of the traditional two-step nested RT-PCR assay and approximately tenfold lower than that of the qRT-PCR assay. When testing the reference strain EV71, this assay showed favorable detection reproducibility and no obvious cross-reactivity. The testing results of 100 clinical throat swabs from HFMD-suspected patients revealed that 41 samples were positive for EV71 by both RTN RT-PCR and traditional two-step nested RT-PCR assays, whereas only 29 were EV71 positive by qRT-PCR assay.

P. Niu and S. Qi contributed equally to this study.

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Introduction

Hand, foot, and mouth disease (HFMD) is a common contagious illness that occurs worldwide both sporadically and in epidemics. The syndrome of HFMD includes fever, vesicular eruptions on the hands, feet, and the anterior part of the buccal mucosa and is caused by members of the species *Enterovirus A*, family *Picornaviridae* [1–4].

HFMD affects more than one million children less than 5 years of age and is responsible for the deaths of several hundred children every year, causing widespread concern in society [5–7].

The common etiologic agents for HFMD are enterovirus 71 (EV71) and coxsackievirus A16 (CVA16) [3, 4, 8–10]. EV71 infection is associated more frequently with severe neurologic diseases, such as aseptic meningitis and brainstem and cerebellar encephalitis, which are rarely observed with CA16 infection [3, 4, 8, 11, 12]. Therefore, rapid and sensitive detection of EV71 in the early phase of HFMD infection is important. Current laboratory detection methods for EV71 such as traditional virus isolation and neutralization are insensitive, labor-intensive and time-consuming. Nucleic acid amplification techniques such as reverse transcription PCR (RT-PCR) [12, 13] and reverse transcription-quantitative PCR (qRT-PCR) [14–17] take less time (2 to 3 h) and are generally used due to their high sensitivity and specificity. Although a few commercial qRT-PCR-based diagnostic kits for EV71 are available for HFMD pathogen surveillance in China, their detection limit is not adequate for assessing clinical specimens with a low viral load in the early phase of HFMD, which can lead to a false-negative diagnosis. The two-step nested RT-PCR assay has sufficient sensitivity, but it is time-consuming and susceptible to cross-contamination during the experiment [18, 19], making it unsuitable for HFMD pathogen surveillance.

In the present study, we developed a real-time nested RT-PCR (RTN RT-PCR) assay in a single closed tube. This assay exhibits superior sensitivity to TaqMan probe-based real-time PCR (qRT-PCR) and has the advantages of low cost and easy performance. In the light of the ongoing endemic spread of HFMD in China, this assay will contribute to enhanced HFMD surveillance in budget-limited rural clinics of provincial and municipal regions in China and aid in the detection of EV71 pathogen in patients with suspected HFMD in the early phase.

Materials and methods

Viruses and clinical samples

This project was approved by the Institutional Review Boards of the Centre of Disease Control and Prevention of China, the Ethical Review Committee of the Institute for Viral Disease Control and Prevention, Center for Disease Control and Prevention of Hebei. Individual written informed consent was obtained from the parents or guardians of all of the participants.

The EV71 subgenotype C4 isolate (strain FY17.08/AN/CHN/2008, GenBank accession no. EU703812), with an infectivity titer of 10^7 50 % tissue culture infective doses ($TCID_{50}$)/ml on human rhabdomyosarcoma (RD) cells, was used as a reference virus to evaluate the sensitivity of the RTN RT-PCR assay. A CVA 16 isolate (strain FY18/AN/CHN/2008, GenBank accession no. EU812514) and other positive clinical samples containing RNA viruses commonly found in the respiratory tract were used as controls to determine the specificity of the ORTN RT-PCR assay. The panel of controls was described previously [20] and included CVA16, human coronavirus NL63, human coronavirus 229E, human coronavirus OC43, human coronavirus HKU1, influenza virus A and B (FluA and FluB), human rhinovirus (HRV), respiratory syncytial virus (RSV), parainfluenza viruses 1, 2, 3 and 4 (PIV1-4), adenovirus (ADV), human metapneumovirus (hMPV), and human bocavirus (HBoV). The control and reference isolates were all obtained from the National Laboratory for Poliomyelitis (NLP), National Institute for Viral Disease Control and Prevention (IVDC), Chinese Center for Disease Control and Prevention (CDC). A total of 100 throat swabs were selected from the collection from during hospitalized children or outpatients under 5 years old diagnosed with HFMD during 2014. All of the specimens and clinical data were collected and provided by staff members of Hebei Center for Disease Control and Prevention, China. This entire study was approved by the local ethics committee. The parents or grandparents of each child in the study provided informed consent for sample collection.

Nucleic acid extraction

Nucleic acid from 140 μl of each reference virus stock, control, and clinical throat swab was extracted using a QIAamp Viral RNA Mini Kit according to the manufacturer's instructions. The extracts were eluted in 30 μl of DNase and RNase-free water and stored at −80 °C until use.

RTN RT-PCR primer design

One-step nested primer sets for EV 71 were derived from previously published two-step nested primers with slight modifications [18]. The inner primers (R2, F2) of both nested PCR assays were the same, while the outer primers (R1, F1) of both nested PCR assay were 19 nucleotides (nt) longer. The outer primer sets (R1, F1) used in the one-step nested PCR maintained the 21-nt sequences of the original outer primer sets for two-step nested PCR but had an additional 19 nt at the 3' ends of the original primers. The specificity of the new outer primers was extensively analyzed *in silico* based on the EV 71 genome sequences currently available in GenBank. The strategy for primer design was to maximize the difference in the annealing

temperatures (64 °C versus 52 °C) of the outer and inner primer sets. This allowed one-step nested amplification to be carried out via temperature switch PCR (TSP) [21]. The one-step nested primer sequences and working concentrations as well as the primers and the probe for qRT-PCR are listed in Table 1. A schematic description of the primer design is shown in Figure 1.

RTN RT-PCR and melting curve analysis

One Step RT-PCR Enzyme Mix (QIAGEN, USA) was used in the construction of the assays for detection of EV71. In a 25-μl reaction system, 5 of μl 5× PCR buffer, 2.5 μl of enzyme mix, 1 mM dNTP mix, 0.7 μM SYTO 9 (Life Technologies, USA), and 2 μl of template nucleic acid were added. Nested primers were added in a 10× mix. Final working concentrations are listed in Table 1.

RTN RT-PCR for EV71 was carried out using a CFX96™ Real-Time PCR System (Bio-Rad, USA) under the following conditions: a 45-min reverse transcription step at 42 °C, a 10-min denaturation step at 95 °C, and 20 cycles of 94 °C for 30 s, 64 °C for 30 s and 72 °C for 30 s, followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s. The assays for dissociation were done by incubating the reaction mixture at 65 °C for 5 s and then increasing the temperature to 95 °C over a period of 20 min. Positive and negative controls were included in each run. The PCR products were analyzed and confirmed by agarose gel electrophoresis (2.0 % agarose gels) to ensure that no undesirable DNA bands were seen and that only the product of the predicted size was found.

Two-step nested RT-PCR and qRT-PCR assays

The two-step nested RT-PCR was carried out as a reference as described [18]. The commercial qRT-PCR assay of EV 71 was also carried out using a CFX96™ Real-Time PCR System (Bio-Rad, USA) under the following conditions: a 45-min reverse transcription step at 42 °C, a 10-s denaturation step at 95 °C, and 40 cycles of 95 °C for 10 s and 62 °C for 45 s. Positive and negative controls were included in each run.

Sensitivity of the RTN RT-PCR assay

To determine the analytical sensitivity of the assay, tenfold serial dilutions (ranging from 10^{-1} to 10^{-8}, equal to 10^{6} $TCID_{50}$/ml to 0.01 $TCID_{50}$/ml) of viral RNA preparation from a reference EV71 isolate were analyzed in triplicate by the RTN RT-PCR assay as described above. For comparison, a TaqMan probe real-time RT-PCR assay and a traditional two-step nested PCR assay were also performed in parallel.

Table 1 Primers designed for the RTNRT-PCR assay, the two-step nested RT-PCR assay, and the qRT-PCR assay

Primer	Sequence	Primer lengths (bp)	Product size (bp)	Product *Tm* (°C)	Concentration[a] (nM)	References
ORTN F1	5'GCAGCCCAAAAGAACTTCACAATGAAATTGTGTAAGGATG3'	40			15	
ORTN R1	5'TAGCATTTGATGATGCTCCAATTTCAGCAGCTTGGAGTGC3'	40	247	83.5±0.5	15	
ORTN F2	5'CTGGAACCTTACCTGTGTCCA3'	21			240	
ORTN R2	5'CCATCCAGGGAGATAGGGTAG3'	21	144	83±0.5	240	[18]
Outer primer F	5'GCAGCCCAAAAGAACTTCAC3'	21				
Outer primer R	5'ATTTCAGCAGCTTGGAGTGC3'	21	247			[18]
Inner primer F	5'CTGGAACCTTACCTGTGTCCA3'	21				
Inner primer R	5'CCATCCAGGGAGATAGGGTAG3'	21	144			
Primer	5'TGATTGAGACACGSTGTGTYCTTA3'	24			480	
Primer	5'CCCGCTCTGCTGAAGAAACT3'	20			480	
Probe	HEX-TCGCACAGCACAGCTGAGACCACTC-BHQ1	25			240	[3]

[a] Primer concentrations in a 25-μl PCR mixture

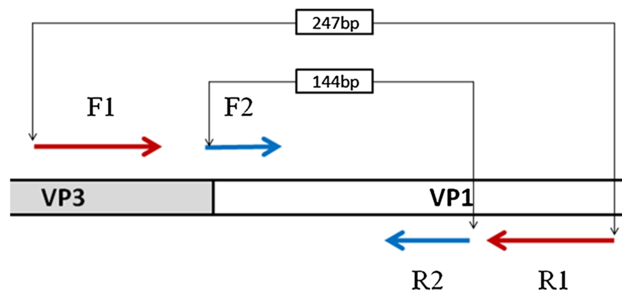

Fig. 1 Schematic description of the primer design for the RTN RT-PCR assay. F1 and R1 are the outer primers with 40 nt in length, which has an annealing temperature of 64 °C. F2 and R2 are the inner primers with 21 nt in length, which has an annealing temperature of 52 °C. For primer sequences, see Table 1

Specificity of the novel RTN RT-PCR assay

To determine the specificity of the assay, viral RNA preparations from a panel of controls were analyzed in triplicate by the novel RTN RT-PCR assay as described above. The panel included CVA16 and other DNA/RNA viruses commonly found in the respiratory tract.

Evaluation of the clinical performance of the novel RTN RT-PCR assay

A total of 100 throat swabs samples were used for the clinical evaluation of the RTN RT-PCR assay for the detection of EV71 according to the protocol described above. For comparison, traditional two-step nested RT-PCR assay and qRT-PCR assay were also performed in parallel. Sequencing was used to resolve discrepant results among the three assays.

Results

Specificity of the nested primer sets and *Tm* of PCR products

As expected, the nested primer sets amplified only the reference EV71, with a single peak in the dissociation plot and no cross-reaction with the other RNA/DNA viruses in the panel of control.

The melting temperature (*Tm*) is defined as the temperature at which 50% of the DNA amplicons are in a double-stranded configuration [22]. The *Tm* value for each amplicon is listed in Table 1. As the *Tm* determines the specificity of the PCR products in the melting curve analysis, the positive samples (144-bp product of the inner PCR) generated an obvious peak in the dissociation plot at 83 ± 0.5 °C, while the negative controls produced a peak in the dissociation plot at 74 °C (primer dimer), and no amplification or dissociation profile at 83 ± 0.5 °C was observed (Fig. 2A). The results of melting curve analysis were further verified by 2 % agarose gel electrophoresis (Fig. 2B).

Sensitivity of the RTN RT-PCR assay

The RNA of reference EV71 with a tenfold serial dilution (equal to 10^6 $TCID_{50}$/ml to 0.01 $TCID_{50}$/ml) was extracted and tested in triplicate to determine the endpoint dilution at which the positive amplification signal (peak in the dissociation plot) was obtained from all the three replicates in the RTN RT-PCR assay. The qRT-PCR results were defined as positive if the *Ct* value was not higher than 35. The limit of detection for the RTN RT-PCR assay was 0.01 $TCID_{50}$/ml with a *Ct* value of 38.3 and shown to be equal to that of the traditional two-step nested RT-PCR assay and approximately tenfold lower than that of the qRT-PCR assay. The results of both RTN RT-PCR and two-step nested RT-PCR were confirmed by agarose gel electrophoresis (data not shown). The standard curve of the RTN RT-PCR assay is shown in Figure 3.

Clinical evaluation

A total of 100 throat swabs collected from patients clinically diagnosed with HFMD were tested by the RTN RT-PCR assay, two-step nested RT-PCR assay, and qRT-PCR assay. The results are shown in Table 2. When 100 samples were tested, both the RTN RT-PCR assay and the two-step nested RT-PCR assay detected 41 (41 %) EV71-positive samples, while only 29 (29 %) samples were EV71 positive, with *Ct* values ranging from 26.5 to 35 by the qRT-PCR assay. The 59 clinical specimens testing negative for EV71 by the RTN RT-PCR assay and two-step nested RT-PCR assay were tested using our previously reported assay (a two-tube multiplex reverse transcription PCR assay for simultaneous detection of sixteen human respiratory virus types/subtypes [20]).

Discussion

The development of PCR techniques has contributed significantly to laboratory diagnosis of EV71 infections in terms of the turnaround time, sensitivity, specificity, and the rate of detection in comparison with the cell culture and neutralization tests. However, traditional PCR methods require amplification in a thermocycler followed by product separation by gel electrophoresis [23], fluorescent capillary electrophoresis [24], or biochip [25], which are time-consuming and laborious procedures. The TaqMan probe real-time RT-PCR assays developed in recent years

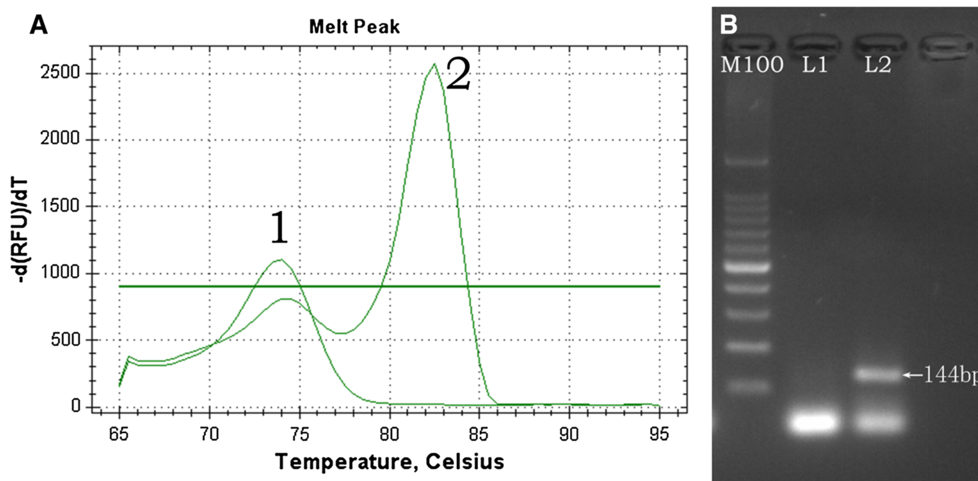

Fig. 2 A. Dissociation plots of the RTN RT-PCR products. The ordinate shows the fluorescence intensity, and the abscissa shows the temperature. 1, the melting curve of the negative control; 2, the melting curve of the positive control; B, the size analysis of the PCR products of the assay; M100, 100-bp DNA ladder marker; Lane 1, negative control; lane 2, 144-bp positive control. The low-molecular-weight bands in lanes L1 and L2 are primer dimers and products of nonspecific amplification

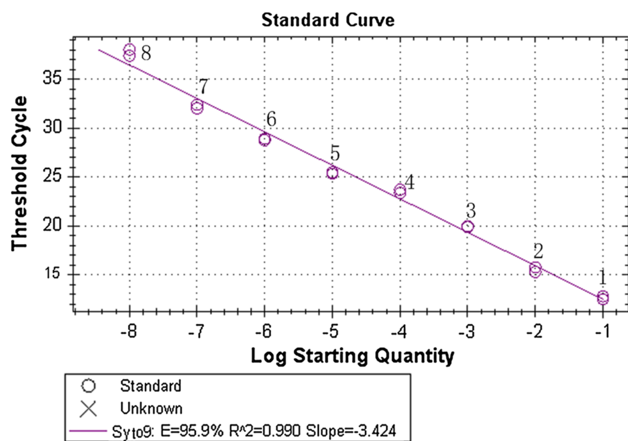

Fig. 3 Standard curve of the RTN RT-PCR assay. Samples 1-8 represent serial tenfold dilutions (log $^{-1}$ to $^{-8}$) of viral RNA containing amounts equivalent to 10^6 - 0.01 $TCID_{50}$/ml

have been routinely performed in well-equipped laboratories for the surveillance of EV71 with improved sensitivity, specificity, and reproducibility [15, 26–28]. However the TaqMan probe real-time RT-PCR assays are expensive in budget-limited rural clinics of provincial and municipal regions in China and the sensitivity is not adequate for the detection of the virus in samples from the early phase of infection, which have a lower viral load [16]. The reported two-step nested PCR is more sensitive than the qRT-PCR assay [3, 18], which was confirmed in the present study. However, an inherent drawback of the two-step nested PCR is the need to open tubes after the first round of amplification to transfer amplicons to a second tube for another PCR amplification, followed by gel electrophoresis, which significantly increases the risk of cross-contamination with amplicons derived from positive specimens during the first round of amplification [29–31]. In the present study, we have adapted and modified a previously described two-step nested RT-PCR [18] using a carefully designed primer and TSP amplification strategy to develop a highly sensitive real-time nested RT-PCR format in a single closed tube that is less prone to cross-contamination, more cost-effective and sensitive.

Previously, we developed a novel multiple PCR platform based on the use of chimeric and fluorescent-dye-labeled universal tag primers and the TSP strategy [32, 33]. In the proposed single-tube RTN RT-PCR assay, the inner primer sets (R2, F2) and outer primer sets (R1,F1) were

Table 2 Comparison of positive and negative results of the RTN RT-PCR, two-step nested RT-PCR and qRT-PCR assays

Assay	No. positive and percentage of EV71	No. negative and percentage of EV71	Ct value
RTN RT-PCR	41(41 %)	59[a](59 %)	26-38
Two-step nested RT-PCR	41(41 %)	59[a](59 %)	26.2-38
qRT-PCR assays	29(29 %)	71(71 %)	26.5-35

[a] The 59 clinical specimens were tested using our previously reported assay [20], and the test found ADV in four samples, HboV in three samples, RSV in three samples, and HRV in one sample

intentionally designed so that the length difference between R2 and F2 and between R1 and F1 would be 19 nt, making a significant difference in the annealing temperatures (64 °C for R1 and F1 and 52 °C for R2 and F2) and possibly allowing an independent (individual) reaction during the following amplification. We postulated that TSP amplification includes two steps with different annealing temperatures, and only one band (the amplified 144-bp product of the inner primer sets) of the positive sample would be visualized by gel electrophoresis. The products of PCR in this assay could then be readily detected by melting curve analysis using SYTO9 [34, 35]. To date, few studies have reported nested-PCR performed in a single tube [36–38]. Unlike those described single-tube nested RT-PCR assays using either fluorescent probes [36], gel electrophoresis [37] or spatial separation of the outer and inner primer sets [38], the RTN RT-PCR assay described in this study combines the reaction and detection steps in a single closed tube, which can effectively prevent contamination and eliminates the needs for post-PCR electrophoresis or a fluorescent probe. It only took three hours for the whole test. The cost of the SYTO9 dye (about $380/100 µl, 20,000 reactions, $0.01/reaction) is much lower than that of a fluorescent probe (about $160/primer; 200 reactions, $0.8/reaction). Therefore, the proposed assay offers the advantage of being a real-time, faster, cost-effective, contamination-free and highly sensitive assay. More detailed comparison of RNT RT-PCR with other available methods is summarized in the supplementary material (Table S1)

The working concentration of each primer, the reaction parameters, and the running conditions of the RTN RT-PCR assay were optimized, and this enabled the detection of EV71 with extremely high sensitivity while maintaining good specificity. The reported sensitivity of the commercial qRT-PCR kit was 0.1 $TCID_{50}$/ml [3], while the sensitivity of the RTN RT-PCR was demonstrated to be 0.01 $TCID_{50}$/ml, which is equal to that of the two-step nested RT-PCR assay and 10 times more sensitive than qRT-PCR. The specificity of the original two-step nested RT-PCR was tested with RNAs extracted from other enteroviruses, including CA16, CA24, CB2-5, ECV9 and ECV30 [18], and the specificity of the RTN RT-PCR derived from the original two-step nested RT-PCR was extensively analyzed further *in silico* and verified using CVA 16 and other common respiratory viruses to ensure accurate detection of EV71.

This RTN RT-PCR assay was further evaluated and compared with two-step nested RT-PCR assay and qPCR assay using 100 clinical specimens. As shown in Table 2, both the RTN RT-PCR and the two-step nested RT-PCR assay detected a total of 41 (41 %) EV71-positive samples with the *Ct* values ranging from 26 to 38, which is superior to the qRT-PCR assay, in which only 29 (29 %) samples were EV71 positive, with the *Ct* values ranging from 26.5 to 35. The 12 samples that were positive by nested RT-PCR but negative by qRT-PCR were confirmed as true positives by sequencing (data not shown). This result suggested the RTN RT-PCR assay possessed higher sensitivity than the qRT-PCR assay and retained the sensitivity of the two-step nested RT-PCR while simplifying the experimental procedures and reducing the handling time.

Additionally, in testing the clinical samples on other different real-time PCR machines (Roche LightCycler 480, ABI 7900HT), we found that the *Tm* values for the amplicons were very similar but not identical to those that we describe. However, the peaks were still well defined, indicating a potential wide adaptability of this assay.

Conclusion

In summary, this RTN RT-PCR assay exhibits extremely high sensitivity and specificity. More importantly, this assay is affordable and convenient for routine use in primary laboratories. The assay is more sensitive than qRT-PCR and has the potential to be widely used as a platform for the rapid screening of EV71 and other virus infections in the early phase in budget-limited rural clinics of provincial and municipal regions in China.

Compliance with ethical standards

Informed consent All aspects of the study were performed in accordance with national ethics regulations and appraised by the Institutional Review Boards of the Center for Disease Control and Prevention of China. Children's parents were apprised of the study's purpose and of their right to keep information confidential. Written informed consent was obtained from parents or caregivers.

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Conflict of interest All authors declare that they have no competing interests.

Disclaimers The views expressed in this article are those of the authors and do not necessarily represent the views of the Chinese Center for Disease Control and Prevention.

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