

A Sensitive and Specific One-step TaqMan RT-qPCR Assay for Rapid Detection of Enterovirus C99

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Abstract

Background

Enterovirus C99 (EV-C99) is an emerging member of the *Enterovirus C* with an expanding geographic distribution and documented cross-species transmission. Current detection methods, including conventional and nested RT-PCR, are limited by prolonged turnaround times and increased risk of cross-contamination which causes false-positive results. This study aimed to develop a rapid, sensitive, and specific assay for EV-C99 detection to further support epidemiological investigations.

Methods

A one-step TaqMan one-step RT-qPCR assay targeting a conserved region of the VP1 gene of EV-C99 was designed and optimized. Specificity of the method was evaluated against multiple enteroviruses, including Coxsackievirus A8, Coxsackievirus A24, Echovirus 6, Poliovirus, EV-C96, and EV-D68. Sensitivity, linearity, and intra-assay repeatability were determined using 10-fold serial dilutions of a plasmid standard (3.66×10^5 to 3.66×10^{-1} copies/ μ L). The assay was subsequently applied to 92 enterovirus-positive wastewater samples collected during 2024–2025, and positive results were validated by Sanger sequencing of VP1.

Results

The developed assay demonstrated high specificity, with amplification observed exclusively for EV-C99. The limit of detection (LOD) was 3.66×10^{-1} copies/ μ L, representing a 100-fold increase in sensitivity compared with conventional RT-PCR. The standard curve exhibited good linearity across the dynamic range, and intra-assay coefficients of variation (CV) ranged from 0.64% to 2.19%. The total assay completed within approximately 2 hours, significantly shorter than conventional RT-PCR time of about 4–6 hours. Among 92 wastewater samples, one (1.09%) was tested positive for EV-C99, and this result was confirmed by conventional RT-PCR and sequencing.

Conclusions

In this study, we established a rapid, sensitive, and specific one-step TaqMan RT-qPCR assay for EV-C99 detection. The method provides a reliable tool for laboratory diagnosis and environmental surveillance of EV-C99, facilitating improved monitoring of EV-C99 circulation.

1. Background

Enteroviruses (EVs) are among the most prevalent human pathogenic viruses. They belong to the family *Picornaviridae*, genus *Enterovirus*, are non-enveloped viruses with single-stranded positive-sense RNA genome [1]. According to the International Committee on Taxonomy of Viruses (ICTV), human enteroviruses are classified into four species, including Enterovirus A-D (EV-A to EV-D), which comprise more than 300 genotypes [2]. Enterovirus C (EV-C), for instance, includes 23 genotypes, such as poliovirus (PV1–PV3), certain Coxsackievirus A types (CVA1, CVA11, CVA13, CVA17, CVA19–CVA22, CVA24), and recently characterized genotypes such as EV-C95, EV-C96, and EV-C99 [3].

The extensive genetic diversity of EVs results from two principal intrinsic mechanisms. First, the error-prone RNA-dependent RNA polymerase (RdRp) lacks proofreading activity, leading to high mutation rates and the formation of quasi-species populations [4–6]. Second, frequent recombination between co-circulating strains further drives viral evolution [7]. Notably, recombination among EV-C members and poliovirus is of particular concern, as it may alter virulence and transmissibility, thus posing challenges to global polio eradication [8].

Enterovirus C99 (EV-C99) was first identified in 2000 from acute flaccid paralysis cases in Bangladesh [9]. Since then, it has been detected in humans and nonhuman primates [10], as well as other mammals [11], across multiple countries [12, 13]. The broad host range suggests possible cross-species transmission and sustained environmental circulation. Given its evolutionary potential and epidemiological distribution, reliable surveillance tools for EV-C99 are in urgent need.

Currently, EV-C99 detection relies primarily on VP1-targeted conventional [14] and nested RT-PCR [15]. These methods require post-amplification processing, thus are relatively time-consuming. In addition, the increased contamination risk also results in false-positive diagnosis. In this study, we developed and validated a one-step TaqMan RT-qPCR assay targeting the VP1 coding region of EV-C99. This method enables rapid, sensitive, and specific detection and quantification of EV-C99, addressing the limitations of conventional approaches while suitable for environmental and clinical surveillance.

2. Methods

2.1 Viral Strains and Sample Sources

All enterovirus strains used for cross-reactivity testing, including Coxsackievirus A8 (CVA8, species A) (GenBank accession No. KP765687.1), Coxsackievirus A24 (CVA24, species A) (GenBank accession No. JX473471.1), Echovirus 6 (E6, species B) (GenBank accession No. KF487190.1), Poliovirus (PV, species C) (GenBank accession No. AF518082.1), EV-C96 (species C) (GenBank accession No. KF495604.1), EV-D68 (species D) (GenBank accession No. PX397553.1) and EV-C99(species C) (GenBank accession No. PX754292), were stored in the strain repository of the Poliomyelitis Laboratory at the Institute of Pathogenic Microorganism Testing, Guangdong Provincial Centre for Disease Control and Prevention (GDCDC). A total of 92 wastewater samples were collected among January 2024 to December 2025

from municipal sewage treatment plants across Guangdong Province, as part of the national acute flaccid paralysis (AFP) surveillance system and environmental poliovirus surveillance program.

2.2 Primer and probe design

The complete genomic sequences of enterovirus C99 (EV-C99) (GenBank accession No.: EF015012.1, MT641390.1, MT641378.1) were retrieved from the GenBank database and aligned using BioEdit 7.2.5 (Ibis Biosciences, Carlsbad, CA, USA). The VP1 coding region was extracted based on the annotated coordinates of the reference strain (GenBank accession No. MT641378.1). Within a relatively conserved region of the VP1 coding sequence, primers and a 22 bp probe were designed using Primer Premier 5.00 (PREMIER Biosoft International, Palo Alto, CA, USA) (Table 1) to detect a 120-bp fragment of the VP1 gene. The selected primers and probe were then evaluated for GC content, annealing temperature, and the presence of hairpin structures using OligoCalc (Northwestern University, Evanston, IL, USA; <https://oligocalc.eu/>). The Ct values obtained from each reaction were plotted against the log of the number of template copies and viral loads were calculated according to the linear equation: $y = -3.490x + 63.03$, with R^2 of 0.998. The positivity cut-off was set at $Ct \leq 35$. For conventional RT-PCR validation, the primer pair 494/497[16] targeting the VP1 region of EV-C was used to generate a 1,080 bp amplicon.

Table 1
Primers and probe designed targeting the VP1 gene of EV-C99.

Primer name	Purpose	Sequence (5'-3')
EV-C99-F	qRT-PCR	CTCACAACCWACACRGCCAG
EV-C99-R		ATGCCACTGCYTGTCCYGAG
EV-C99-P		FAM-CCCAGCRCAAGYGGCGTGAAYA-BHQ-1
494 [16]	RT-PCR	GAYGAYWSITTIACIGAIGGNGG
497 [16]		GCITTITTITGRTGICCRAANCC

2.3 Preparation of the standard plasmid of the EV-C99 VP1 gene

The VP1 gene of the EV-C99 reference strain (GenBank accession No. MT641378.1) was cloned into the pUC57 vector (2710 bp, Guangzhou IGE Biotechnology, Guangzhou, China) to generate the recombinant plasmid pUC57-EV-C99-VP1 (3618bp). Plasmid DNA was extracted using the EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and its concentration was quantified using a NanoDrop One microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Copy number (copies/μL) =
$$\frac{[Plasmid\ concentration (ng/\mu\ l)] \times 10^{-9} \times 6.023 \times 10^{23}}{660 \times plasmid\ length (bp)}$$

where C is the plasmid concentration (ng/ μ L), L is the plasmid length in base pairs (bp), 660 is the average molecular weight of one base pair of double-stranded DNA (Da), and 6.022×10^{23} is Avogadro's constant. Based on this calculation, the standard copy number was determined to be 3.66×10^{10} copies/ μ L.

2.4 Optimization of the TaqMan qRT-PCR method

The One-step RT-qPCR was performed in a 25 μ L reaction volume using the One Step PrimeScript™ RT-PCR Kit (TaKaRa Bio Inc., Kusatsu, Shiga, Japan; Cat. No. RR064A).

To optimize the reaction, three combinations of primers and probe volumes were tested using 10 μ mol/L stock solutions: (i) 1.0 μ L each primer and 2.0 μ L probe (Combination 1); (ii) 1.0 μ L each primer and 0.5 μ L probe (Combination 2); and (iii) 0.75 μ L each primer and 0.5 μ L probe (Combination 3) (Table 2). The thermal cycling condition was as follows: Uracil N-Glycosylase (UNG) pretreatment at 25°C for 10 min to eliminate carryover contamination; reverse transcription at 50°C for 10 min; initial denaturation at 95°C for 30 s; followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 50°C for 30 s, and extension at 60°C for 30 s. Fluorescence data were collected during the extension step. All qPCR experiments were performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA).

Table 2
Primers and Probe Concentration Recombination

Combination	Probe (μ L)	Forward primer (μ L)	Reverse primer (μ L)
1	0.8	0.4	0.4
2	0.2	0.4	0.4
3	0.2	0.3	0.3

2.5 Specificity testing

To evaluate the specificity of the developed assay, seven enterovirus strains were tested, including Coxsackievirus A8 (CVA8, species A), Coxsackievirus A24 (CVA24, species A), Echovirus 6 (E6, species B), Poliovirus (PV, species C), EV-C96 (species C), EV-D68 (species D) and EV-C99 (species C). The pUC57-EV-C99-VP1 plasmid was used as positive control (PC), and nuclease-free water was used as a negative control (NC). Using the qRT-PCR method, amplification curves and cycle threshold (Ct) values were analyzed via CFX Maestro 2.3 (Bio-Rad Laboratories, USA). Samples were considered positive when a distinct S-curve was observed with a Ct value ≤ 35 .

For comparative analysis, conventional RT-PCR [16] was performed in parallel with the TaqMan RT-qPCR assay for specificity evaluation using the SuperScript IV UniPrime One-Step RT-PCR System (Invitrogen, Waltham, MA, USA; Cat. No. 12596100). PCR products were visualized by 2% agarose gel electrophoresis.

2.6 Sensitivity Testing

The pUC57-EV-C99-VP1 plasmid was diluted with nuclease-free water, generating a dilution series ranging from 3.66×10^5 to 3.66×10^{-2} copies/ μL . Each dilution was prepared in triplicate, and 2 μL of each dilution was used as a template for one-step RT-qPCR under the optimized reaction conditions. Each run included the EV-C99 plasmid solution (3.66×10^5 copies/ μL) as a positive control (PC) and nuclease-free water as a no-template control (NC). The limit of detection (LOD)[17] was defined as the lowest concentration yielding positive amplification ($C_t \leq 35$) in all replicates across three independent runs (100% detection rate).

The same 10-fold serial dilutions of pUC57-EV-C99-VP1 plasmid (3.66×10^5 to 3.66×10^{-2} copies/ μL) used for TaqMan RT-qPCR LOD determination were subjected to conventional RT-PCR [16]. The lowest concentration yielding a visible band on agarose gel was recorded as the LOD for conventional RT-PCR.

2.7 Repeatability testing

The serially pUC57-EV-C99-VP1 plasmid dilutions were also used in the repeatability test. The C_t values for each replicate were recorded, and the mean, standard deviation (SD), and coefficient of variation (CV) were calculated to evaluate the stability of the RT-qPCR system. CV (calculated as: $CV = (SD/\text{mean}) \times 100\%$) is used to compare the degree of variation across different concentration levels. A lower CV value indicates better relative reproducibility of the method. All 92 wastewater samples were simultaneously analyzed by conventional RT-PCR [16]. PCR products were visualized by agarose gel electrophoresis, and positive samples were confirmed by Sanger sequencing of the VP1 region.

2.8 Testing of clinical samples

The developed TaqMan RT-qPCR assay was applied to 92 wastewater samples collected between January 2024 and December 2025 from municipal sewage treatment plants in Guangdong Province, China. All samples had been initially screened as enterovirus-positive using a Pan-Enterovirus Universal Nucleic Acid Detection Kit (SciGene Biotechnology, Shenzhen, China, Cat. No. 20193400094), but remained untyped at the serotype level. The pUC57-EV-C99-VP1 plasmid was applied as a positive control (PC), while nuclease-free water served as the no-template control (NC). To validate the reliability of the qPCR assay, all samples were concurrently analyzed by Sanger sequencing.

The resulting sequences were submitted to the Enterovirus Genotyping Tool (software v2.23.1, National Institute for Public Health and the Environment [RIVM], Bilthoven, the Netherlands, <https://www.rivm.nl/mpf/typingtool/enterovirus/>) and the NCBI BLAST (National Center for Biotechnology Information, <https://blast.ncbi.nlm.nih.gov/>) server for serotype identification, allowing comparison with the qPCR results. The phylogenetic analysis was performed using MEGA-X 10.2.6 (Molecular Evolutionary Genetics Analysis, Tempe, AZ, USA), and the phylogenetic tree was constructed based on the neighbor-joining method, with the reliability of the topological structure assessed using 1,000 bootstrap replicates.

3. Results

3.1 Optimization of Reaction Conditions

Using the reaction system recommended in the Probe qPCR Mix (with or without UNG) kit manual and EV-C99 plasmid standards, the primer-probe concentrations were optimized. Three different primer-probe combinations were evaluated in 25 µL reaction volumes (Table 1).

During amplification, primer combination 1 exhibited the strongest fluorescence signal, the earliest Ct value, and the most stable plateau phase of the amplification curve (Fig. 1), indicating that the probe concentration was sufficient even for detection of low-copy samples. Therefore, this primer combination (combination 1) was selected as the optimal setup, with final concentrations of 0.4 µmol/L for primers and 0.8 µmol/L for the probe.

Orange curve refers to primer combination 1, green curve refers to primer combination 2, blue curve refers to primer combination 3.

3.2 Specificity

Specificity was evaluated using seven different enteroviruses, including CVA8, CVA24, E6, PV, EV-C96, EV-D68, EV-C99. The pUC57-EV-C99-VP1 plasmid (EV-C99 plasmid standard) served as positive control (PC), and nuclease-free water as negative control (NC). During testing, amplification was observed exclusively for EV-C99, including the plasmid standard and the GDCDC-stored EV-C99 strain. No cross-reactivity was detected with other enteroviruses (Fig. 2A). As a comparison, conventional RT-PCR run on the same panel presented consistent results (Fig. 2B). These results demonstrate that both the developed TaqMan RT-qPCR assay and the former RT-PCR assay are highly specific for EV-C99 detection, with no cross-reactivity against other enteroviruses.

A. Specificity experiment of EV-C99 TaqMan qPCR assay. Green curve: pUC57-EV-C99-VP1, red curve: EV-C99, blue curves: six other enteroviruses and nuclease-free water. B. Specificity of the EV-C99 RT-PCR assay. M: 1000 bp DNA ladder, PC: pUC57-EV-C99-VP1 plasmid, NC: nuclease-free water.

In addition, the TaqMan RT-qPCR assay offers a significantly reduced turnaround time compared with conventional RT-PCR. The established one-step TaqMan RT-qPCR assay required approximately 2 hours from RNA to result, whereas conventional RT-PCR takes roughly 2.5 hours for amplification plus an additional 1 hour for agarose gel electrophoresis and visual interpretation, extending the total assay time to about 4 hours [16], or even a turnaround time of approximately 6–7 hours including setup. These results present a substantial improvement in workflow efficiency of the established assay.

3.3 Sensitivity

Based on the copy number of the EV-C99 standard, eight tenfold serial dilutions were prepared, ranging from 3.66×10^5 to 3.66×10^{-2} copies/µL. TaqMan qPCR was then performed to test sensitivity of the assay. The amplification curves were monitored and the corresponding Ct values were recorded

accordingly. All three replicates at 3.66×10^{-1} copies/ μ L gave clear amplification curves (Fig. 3A), further indicating the limit of detection (LOD) of this assay.

For comparison, conventional RT-PCR on the same dilution series only produced visible bands down to 3.66×10^1 copies/ μ L on agarose gel electrophoresis (Fig. 3B). No amplification was observed in the NC for either method. Thus, these results indicate that the one-step TaqMan qPCR assay is more sensitive, outperforming conventional RT-PCR especially for low-copy EV-C99 samples.

A. TaqMan RT-qPCR amplification curves of 10-fold serial dilutions (3.66×10^5 to 3.66×10^{-2} copies/ μ L).
B. Agarose gel electrophoresis of RT-PCR products from the same dilution series. M: marker, PC: positive control, NC: negative control. TaqMan RT-qPCR detected EV-C99 to 3.66×10^{-1} copies/ μ L, whereas conventional RT-PCR showed visible bands only at concentrations $\geq 3.66 \times 10^1$ copies/ μ L.

3.4 Intra-assay Repeatability

Each serial dilution of the standard plasmid was tested in triplicate using the developed TaqMan qPCR assay. The standard deviation (SD) ranged from 0.15 to 0.57, and coefficient of variation (CV) from 0.64% to 2.19% (Table 3), indicating good intra-assay repeatability and high precision of the established method across the linear range. These results indicate high precision and robust reproducibility of the established assay.

Table 3
Repeatability Experiment of EV-C99 TaqMan qPCR

Copies/ μ L	Ct						SD	CV (%)
	1	2	3	Max	Min	Mean		
3.66×10^5	15.80	15.50	15.63	15.63	15.50	15.64	0.15	0.96
3.66×10^4	19.16	19.43	19.62	19.62	19.16	19.40	0.23	1.19
3.66×10^3	22.90	22.29	23.04	23.04	22.29	22.74	0.40	1.75
3.66×10^2	26.17	26.65	25.51	26.65	25.51	26.11	0.57	2.19
3.66×10^1	29.88	30.03	29.65	30.03	29.65	29.85	0.19	0.64
3.66×10^0	33.69	34.15	33.18	34.15	33.18	33.67	0.49	1.44
3.66×10^{-1}	36.15	36.73	36.91	36.15	36.91	36.60	0.40	1.09

3.5 Sample Testing and Viral Genotyping

The developed TaqMan qPCR assay was further applied to 92 sewage samples collected among 2024 to 2025 in Guangdong province. These samples had been preliminarily tested as enterovirus (EV)-positive but remained ungenotyped. Reference standards were used as positive controls, while nuclease-free

water served as the negative control. To validate the reliability of the established RT-qPCR assay, all clinical samples were then analyzed by Sanger sequencing.

The results showed that one sample (designated as 4540/GD, original laboratory No. 912) was tested positive for EV-C99 among the 92 samples (Fig. 4A), giving a positivity rate of 1.09% (1/92). Both TaqMan RT-qPCR and conventional RT-PCR identified the same single positive sample (Fig. 4B), with a complete concordance (100%) between the two methods.

A. Detection of clinical samples using qRT-PCR assay. Red curve: PC, green curve: the positive test sample (No. 912). B. Agarose gel electrophoresis of conventional RT-PCR products. M: DNA marker (1000 bp), PC: positive control.

For further confirmation, the VP1 region of all clinical samples were sequenced by Sanger sequencing, and the resulting sequences were analyzed using an enterovirus typing tool together with BioEdit for genotype assignment (Fig. 5). Although only one positive sample was identified, these findings preliminarily validate the established assay's suitability for environmental surveillance.

The phylogenetic tree was constructed using MEGA-X with 1,000 bootstrap replications and displayed in a circular layout. Branches are color-coded by genotype (A-M). The red arrow indicates the sequence obtained in this study (4540GDCHN/2024). Reference sequences were retrieved from the GenBank database, with accession numbers, countries of origin, and collection years annotated. The outgroup (Coxsackievirus A24, AF081311.1) is shown in grey.

4. Discussion

Enteroviruses (EVs) are among the most common pathogens causing human enteric infections, capable of inducing a wide spectrum of diseases and posing significant public health challenge. Their circulation follows distinct seasonal patterns in temperate and tropical regions, and co-infections are frequently reported [18]. Multiple genotypes often co-circulate with overlapping clinical symptoms, making it difficult to distinguish them. Therefore, rapid, genotype-specific diagnostic tools are required for both clinical management and public health surveillance.

Molecular typing of enteroviruses relies on sequencing the VP1 capsid gene, which carries serotype-specific neutralization epitopes and serves as the gold standard for serotype identification [19]. This approach has enabled the identification and tracking of individual strains across diverse geographic regions.

Among the numerous EV-C serotypes, EV-C99 has been identified globally [20]. Since its first identification in 2000 in Bangladesh [9], EV-C99 has exhibited distinct regional epidemiological patterns, with prevalence reported in Africa [10, 11, 21, 22], Asia [13, 23, 24], Europe, and South America [12, 15, 25]. Africa has the highest detection rate. In addition, besides its primary host humans, the virus has also

been detected in non-human primates and other mammals [10, 11]. Therefore, its potential for cross-species transmission requires better surveillance.

Traditional enterovirus detection still relies primarily on viral isolation using cell culture with observation of cytopathic effects (CPE), followed by serum neutralization tests for serotype identification. These methods are associated with lengthy turnaround times (typically 1–2 weeks), labor-intensive workflows, and low sensitivity [26], thereby limiting their utility for rapid diagnosis and timely implementation of outbreak control measures [27]. Hence, the development of a rapid, serotype-specific assay for EV-C99 is urgently needed.

Real-time quantitative PCR (qPCR) offers high sensitivity, rapid turnaround, and reliable quantification, thus is now widely applied in viral diagnostics [28]. Several pan-enterovirus qPCR assays targeting the 5' untranslated region (5'UTR) have been developed for genus-level screening [29–31]. However, the 5'UTR is highly conserved across enterovirus serotypes, precluding serotype-specific identification [32, 33].

In contrast, the VP1 coding region encodes the primary capsid protein and contains serotype-specific neutralizing antigenic epitopes. The substantial sequence divergence of VP1 among different serotypes makes it an ideal target for enterovirus serotyping [34, 35]. Molecular typing strategies targeting the VP1 coding region are currently widely adopted reference methods for enterovirus serotyping [36], but their requirement for post-PCR processing limits throughput and increases contamination risk. The TaqMan RT-qPCR assay established in this study has the potential to address these limitations by enabling real-time serotype-specific detection of EV-C99 with improved sensitivity and workflow efficiency, which may be suitable for large-scale environmental surveillance and rapid outbreak response.

This study targeted the EV-C99 VP1 coding region to design specific primers and a TaqMan probe, and established a one-step RT-qPCR assay for rapid, specific, and sensitive detection of EV-C99. Compared to the conventional VP1-targeted RT-PCR method used as a reference in this study, the established TaqMan RT-qPCR assay offers several distinct advantages. Conventional RT-PCR typically takes 4–5 hours for amplification followed by post-PCR processing, including agarose gel electrophoresis and band interpretation [16]. In contrast, the established one-step TaqMan RT-qPCR assay completes amplification and detection in a single tube within approximately 2–3 hours [37]. Sensitivity comparison further highlights the superiority of the RT-qPCR approach. In this study, the LOD of the TaqMan RT-qPCR assay was 3.66×10^{-1} copies/ μL , whereas conventional RT-PCR had an LOD of 3.66×10^1 copies/ μL .

In the specificity evaluation, the EV-C99 standard plasmid was tested alongside nucleic acid from six heterologous enterovirus strains (CVA8, CVA24, E6, PV, EV-C96, and EV-D68). Only EV-C99 yielded positive amplification, whereas all other serotypes were negative, indicating high specificity. Given that the EV-C species comprises multiple serotypes with high sequence similarity in the VP1 region, future studies should expand cross-reactivity testing to include additional closely related serotypes (e.g., CV-A11, CV-A13, CV-A17, CV-A20, CV-A21, EV-C95, and EV-C102) to further define the specificity limits of the assay.

In addition, the TaqMan RT-qPCR assay demonstrated excellent intra-assay repeatability. Using seven 10-fold serial dilutions (3.66×10^5 to 3.66×10^{-1} copies/ μ L), the standard deviation (SD) ranged from 0.25 to 0.57 cycles, and the coefficient of variation (CV) ranged from 0.64% to 2.19%. These low CV values (< 2.2%) across the entire linear range indicate high precision and robustness, consistent with the acceptable performance criteria for diagnostic RT-qPCR assays.

The established assay was further applied to 92 wastewater samples pre-screened as enterovirus-positive. One sample (1.09%) tested positive for EV-C99 by RT-qPCR, and this result was confirmed by Sanger sequencing of the VP1 region. The two methods agreed completely for that single positive sample. However, the limited number of positive samples precludes a robust statistical assessment of analytical sensitivity. Nonetheless, these findings preliminarily validate the assay's reliability for environmental surveillance of EV-C99.

In summary, we developed a one-step TaqMan RT-qPCR assay for rapid, sensitive, and specific detection and quantification of EV-C99. The assay achieves a LOD of 3.66×10^{-1} copies/ μ L, high specificity against heterologous enteroviruses, and excellent intra-assay repeatability (CV < 2.2%). When tested on 92 wastewater samples, the assay identified one EV-C99-positive sample, which was then confirmed by Sanger sequencing. Although the low prevalence of EV-C99 in the tested sample set limited the statistical validation of analytical sensitivity, the method still provides a valuable tool for laboratory diagnosis and environmental surveillance of EV-C99. Future studies should expand cross-reactivity testing to broader EV-C serotypes and validate the assay on a larger panel of clinical and environmental specimens.

Conclusions

In this study, we developed a one-step TaqMan RT-qPCR assay for rapid, sensitive, and specific detection of EV-C99. The assay has a limit of detection of 3.66×10^{-1} copies/ μ L, and a good intra-assay repeatability (CV < 2.2%). When applied to 92 wastewater samples, one sample was tested to be EV-C99 positive, which is further confirmed by Sanger sequencing. The established method offers a practical tool for EV-C99 diagnosis and environmental surveillance. Future work should include a broader range of EV-C serotypes and validation on larger clinical and environmental samples.

Abbreviations

RT-PCR

Reverse Transcription Polymerase Chain Reaction

LOD

limit of detection

RT-qPCR

Reverse Transcription Quantitative Polymerase Chain Reaction

CV

Coefficient of Variation

GDCDC

Guangdong Provincial Centre for Disease Control and Prevention

AFP

Acute Flaccid Paralysis

PC

positive control

NC

Negative Control

SD

standard deviation

EV

Enterovirus

CPE

Cytopathic effect

Declarations

Ethics approval and consent to participate

Not applicable. This study did not involve human participants, human tissue, or identifiable personal data. Wastewater samples were collected from municipal sewage systems for environmental monitoring purposes only. No ethical approval or informed consent was required

Consent for publication

All authors consent for publication.

Competing interests

The authors declare no conflicts of interest.

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Author Contribution

JS, WZ, BL carried out experiments and data analysis, JS wrote the original draft and prepared the figures and tables, BL performed sequencing, WZ, ML, YZ contributed to funding acquisition, QY, YZ contributed to conceptualization, supervision and funding acquisition. All authors read and approved the final manuscript.

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Data Availability

This publication and its supplementary information files include all data presented in this study. The genomic sequences of the viruses isolated in this study have been deposited in NCBI GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) under the accession number PX754292.1.

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Figures

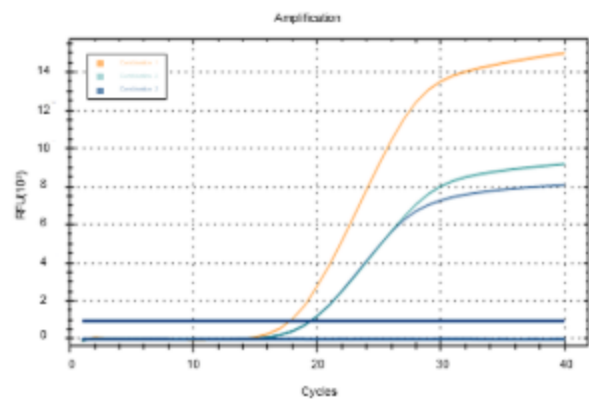


Figure 1
Optimization of EV-C99 TaqMan qPCR Reaction Conditions.

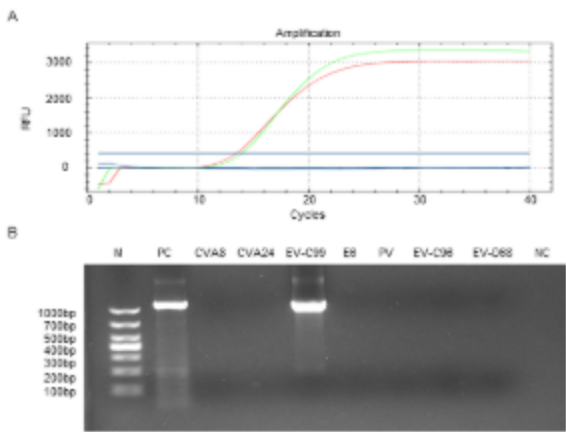


Figure 2
Specificity evaluation of the TaqMan RT-qPCR assay for EV-C99.

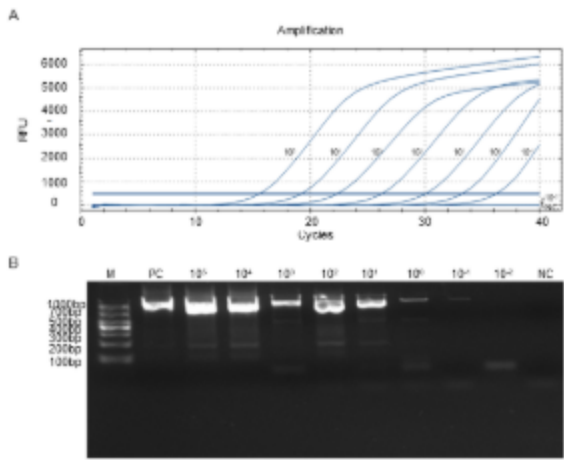


Figure 3

Sensitivity of the TaqMan RT-qPCR and conventional RT-PCR assays.

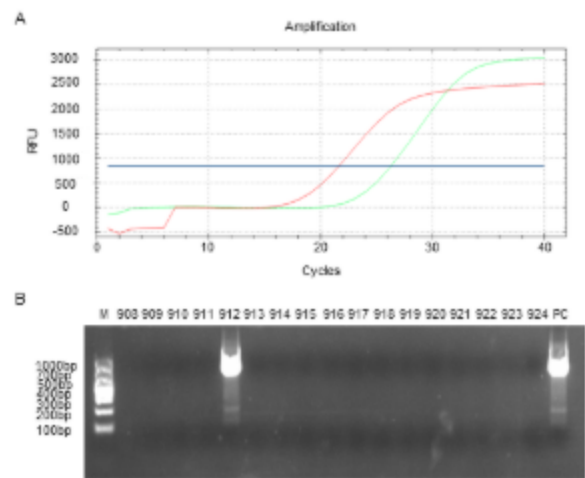


Figure 4

Detection of EV-C99 in clinical samples.

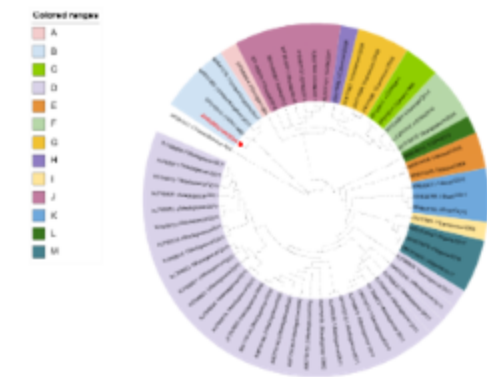


Figure 5

Phylogenetic analysis of EV-C99 (4540/GD) based on VP1 sequences.