American Journal of Plant Biology
2025, Vol. 10, No. 2, pp. 35-40
https://doi.org/10.11648/j.ajpb.20251002.13

Research Article

Establishment of Real-Time Fluorescence Detection Method for Carnation Etched Ring Virus

Wang Jiaying[1, 2], Cui Junxia[1, 2], Sun Jiayu[3], Shen Jianguo[4], Yu Cui[5], Chen Xianfeng[6, *]

[1]Technical Center, Ningbo Customs, Ningbo, China

[2]Technical Center, Ningbo Academy of Inspection and Quarantine, Ningbo, China

[3]College of Biological and Environmental Sciences, Zhejiang Wanli University, Ningbo, China

[4]Technology Center of Fuzhou Customs, Fuzhou, China

[5]Technical Center for Animal & Plant & Food Inspection and Quarantine of Shanghai Customs, Shanghai, China

[6]Animal and Plant Quarantine Office, Ningbo Customs, Ningbo, China

Abstract

Carnation etched ring virus (CERV; genus *Caulimovirus*, family Caulimoviridae) is a destructive plant pathogen subject to stringent global phytosanitary regulations due to its severe impact on carnation production. As a quarantine organism in multiple countries, including Madagascar and Peru, CERV poses significant risks to international horticultural trade, particularly through asymptomatic infections in propagated planting materials. Current diagnostic challenges, such as the limitations of conventional PCR in balancing speed and sensitivity, underscore the urgent need for robust detection tools to prevent transboundary spread. This study aimed to develop a rapid, sensitive, and specific TaqMan-based real-time PCR assay to enhance phytosanitary screening during port inspections. Targeting a conserved region within the CERV genome (GenBank AJ853858.1, positions 1500–1808), two primer pairs and four probes were systematically evaluated to optimize detection efficiency. The finalized assay demonstrated a sensitivity threshold of 3×10^3 copies/μL, comparable to conventional end-point PCR, while significantly reducing processing time. Specificity testing confirmed no cross-reactivity with taxonomically related viruses, including *Carnation ringspot virus*, *Cowpea mosaic virus*, *Cauliflower mosaic virus*, and *Carnation latent virus*, ensuring reliable discrimination. Thermal cycling conditions were streamlined to a 40-cycle protocol with denaturation at 95°C (10 s), annealing at 56°C (15 s), and extension at 60°C (20 s), enabling completion within 90 minutes. This advancement provides a high-throughput solution for regulatory agencies to intercept contaminated consignments efficiently, addressing critical gaps in existing phytosanitary frameworks. By combining rapid turnaround with robust accuracy, the assay strengthens global efforts to safeguard carnation cultivation from CERV-induced losses. Its implementation in trade inspections is particularly vital for detecting latent infections in asymptomatic plant tissues, a major route of pathogen dissemination. The study underscores the importance of molecular innovation in supporting sustainable agriculture and international biosecurity networks, advocating for the integration of such tools into standardized phytosanitary protocols.

*Corresponding author: chenxf@nbciq.gov.cn (Chen Xianfeng)

Received: 2 May 2025; **Accepted:** 13 May 2025; **Published:** 16 June 2025

Keywords

Carnation Etched Ring Virus, Detection, Real-time PCR, Plant Virus

1. Introduction

Carnation (*Dianthus caryophyllus*), a globally significant ornamental species, holds substantially commercial value in the floriculture industry [1]. Viral pathogens, including viruses, viroids, and phytoplasmas, pose persistent threats to the sustainable cultivation of ornamental crops [2]. Among these, *Carnation etched ring virus* (CERV; genus *Caulimovirus*, family Caulimoviridae) is ranked as the second most economically significant viral pathogen affecting carnation production worldwide [3]. First documented in the United Kingdom with unresolved geographic origins, CERV has been detected in all major carnation-growing regions under both greenhouse and field cultivation systems [4, 5]. Its current status as a regulated quarantine pest in Madagascar and Peru underscores its phytosanitary importance. Phylogenetic evidence suggests that the global dissemination of CERV coincided with intensive international trade of carnation propagules prior to the establishment of reliable molecular detection protocols [6]. The strategic implementation of virus-certified planting materials coupled with rigorous phytosanitary protocols demonstrates potential for achieving substantial reductions in global CERV incidence rates [7].

Carnation remains the sole documented natural host of CERV. Experimental transmission studies, however, have demonstrated its capacity to infect selected species within the Caryophyllaceae family, including *Dianthus barbatus* (sweet william), *Silene armeria*, and *Saponaria vaccaria* [8]. CERV infection manifests throughout both vegetative and reproductive developmental stages of carnations [9]. Characteristic foliar symptoms include chlorotic rings, necrotic flecks, and linear patterns that frequently coalesce into expansive necrotic lesions. Coinfection with other carnation-infecting viruses exacerbates symptom severity, inducing systemic chlorosis, stem streaking, and multifocal necrosis [4]. Infected plants exhibit reduced vigor. Tissue-specific viral accumulation analysis reveals preferential localization in photosynthetic and reproductive organs [10]. This distribution pattern establishes foliar and floral tissues as optimal sampling targets for diagnostic assays. The discontinuous spatial distribution of viral particles within host tissues necessitates standardized sampling protocols to ensure detection reliability.

The development of a real-time PCR assay for CERV detection addresses critical limitations inherent in conventional diagnostic methods, such as end-point PCR and enzyme-linked immunosorbent assay (ELISA). While ELISA offers simplicity, its reliance on antibodies compromises sensitivity, particularly for asymptomatic infections with low viral titers, and raises risks of cross-reactivity with related pathogens. End-point PCR, though more sensitive than ELISA, necessitates post-amplification gel electrophoresis, prolonging turnaround time and increasing contamination risks during handling. In contrast, real-time PCR combines rapidity, closed-tube automation, and superior sensitivity, enabling simultaneous amplification and detection of target sequences within 90 minutes—a decisive advantage for high-throughput port inspections. Furthermore, its quantitative capability allows for viral load assessment, critical for gauging infection severity and monitoring latent infections in traded planting materials. By eliminating post-PCR processing and reducing hands-on time, the method minimizes human error while enhancing reproducibility. This approach is particularly vital for intercepting CERV in symptomless propagative stocks, a primary conduit for transboundary spread, thereby aligning with the urgent need for phytosanitary tools that harmonize speed, precision, and scalability in global horticultural trade.

Seasonal fluctuations in symptom expression create epidemiological risks, particularly during winter cultivation cycles [11]. Accurate and timely viral diagnosis constitutes a critical component in integrated disease management strategies for CERV. In this study, a real-time fluorescent PCR detection method for CERV was established based on the conserved genomic region.

2. Materials and Methods

2.1. Sample Collection and Nucleic Acid Extraction

The experimental materials included the plasmid carrying target sequence of CERV, Carnation ringspot virus (CRSV; Agdia, USA), Cowpea mosaic virus (CPMV; Agdia, USA), Cauliflower mosaic virus (CaMV; Agdia, USA), Carnation latent virus (CLV; Agdia, USA), and healthy seedlings. Either total DNA or RNA was extracted using DNeasy Plant Mini Kit (Qiagen, Germany) or RNeasy Plant Mini Kit (Qiagen, Germany) accordingly.

2.2. Method Establishment

Partial nucleic acid region from DNA binding protein (accession No. AJ853858.1, from 1500 to 1808) [12] was selected as the target sequence. Primer design was carried out using primer-BLAST [13]. Primers went through testing via

end-point PCR and one pair was selected, according to which probes were designed. Similarly, the final probe was chosen based on amplification curves and Ct value. Then, amplification procedure was modified to enhance the efficiency.

2.3. Method Parameter Analysis

The specificity and sensitivity of this developed real-time PCR were estimated using collected samples. A series of positive plasmid solutions with concentration ranging from 3×10^7 copies/μL to 3×10^0 copies/μL, 8 gradients, were prepared and subject to real-time PCR with distilled water as negative control, to determine the method sensitivity. Each treatment was conducted in two technical replicates in both analysis.

3. Results

3.1. Method Development and Validation

Altogether 2 primer pairs were generated based on the selected target sequence (Table 1). After testing via end-point PCR, primer pair No. 1 was chosen. Basing on primer pair No. 1, 4 probes (Table 2) were designed and probe No. 4 was chosen according to the amplification efficiency. Amplification was carried out under the following procedure: initial denaturation (95°C, 30 s); 40 cycles of denaturation (95°C, 10 s), annealing (56°C, 15 s), and extension (60°C, 20 s).

Table 1. *Generated primers by primer-BLAST.*

No.	Forward primer				Reverse primer				Product length
	Sequence (5'->3')	Length	Tm	GC%	Sequence (5'->3')	Length	Tm	GC%	
1	GGCCACCATCGCGTCAGAAATC	22 nt	64.34	59.09	CCCATGCCTTCATTTCCGACGTCAAAGT	28 nt	59	50	309 bp
2	GGCCAC-CATCGCGTCAGAAATC	22 nt	64.34	59.09	GTGGCCATTTTAAAGCGTT-GGGATTAGTT	29 nt	56.2	41.3	344 bp

Table 2. *Designed probes based on primer pair No. 1.*

No.	Sequence (5'->3')	Length
1	[FAM]GGATCAACTCCTGAAGAAAGCTCGAATC[BHQ1]	28 nt
2	[FAM]GAAATCCTCAACAAAGACAAAGCTATCATTCC[BHQ1]	32 nt
3	[FAM]CAACTCCTGAAGAAAGCTCGAATC[BHQ1]	24 nt
4	[FAM]CAACTCCTGAAGAAAGCTCGAA[BHQ1]	22 nt

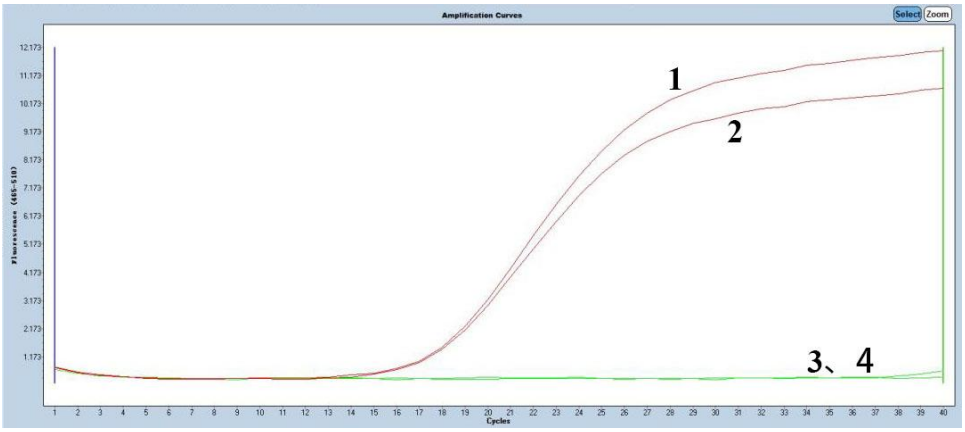


Figure 1. *Method validation results 1-2: Positive plasmid; 3-4: healthy seedlings and NTC.*

Method validation was performed using positive plasmid and healthy seedlings. As shown in Figure 1, specific amplification was achieved with the final primer-probe set, yielding Ct values at 16.88 and 16.71 (duplicates). No amplification curves were observed in healthy seedling samples or no-template controls.

3.2. Parameter Analysis

Sensitivity analysis was conducted using 8 concentration gradients along with the negative control. Concentration lower than 3×10^3 copies/μL failed in amplification (Figure 2). Thus, the sensitivity of this real-time PCR assay was estimated at 3×10^3 copies/μL, comparable to conventional PCR [14].

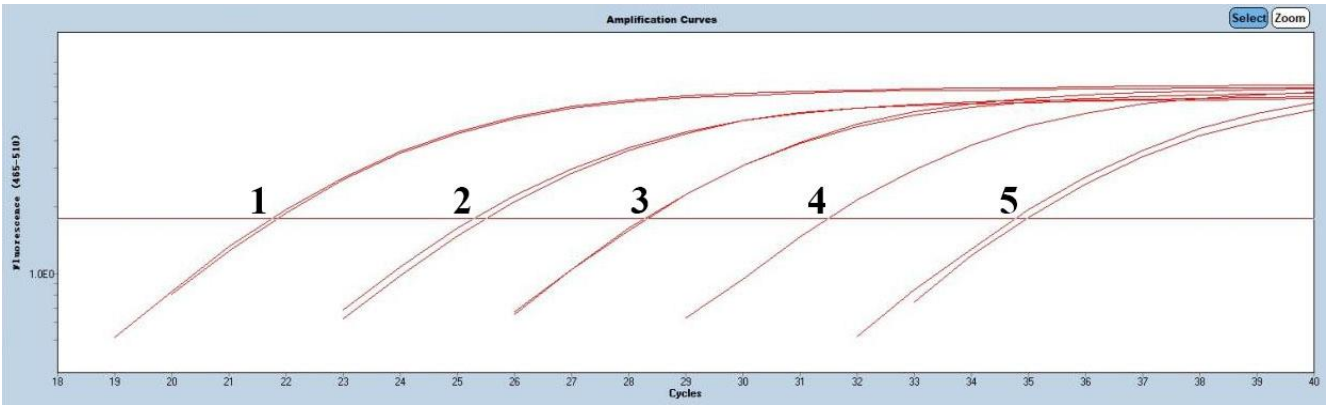
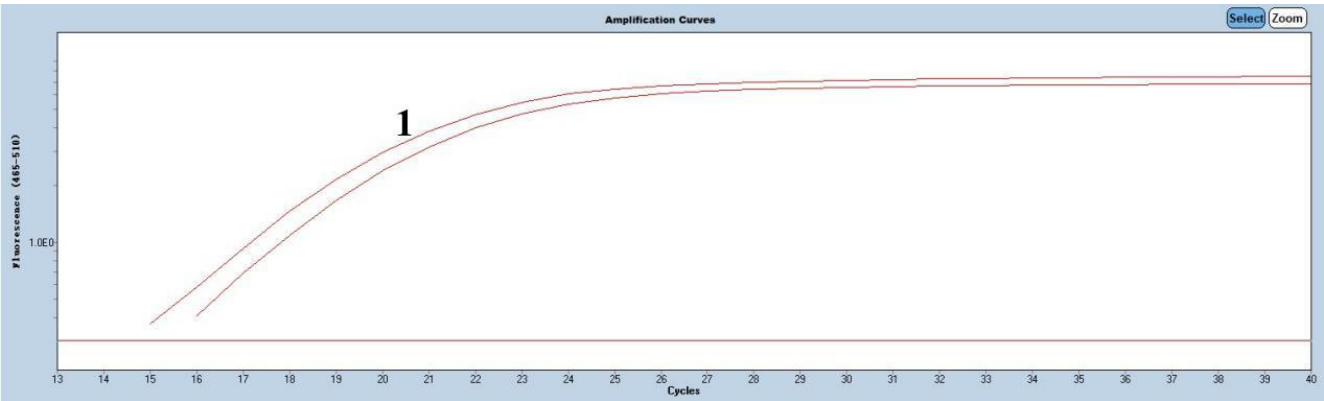


Figure 2. *Sensitivity analysis results.*

1: 3×10^7 copies/μL; 2: 3×10^6 copies/μL; 3: 3×10^5 copies/μL: 4: 3×10^4 copies/μL; 5: 3×10^3 copies/μL; others: 3×10^2 copies/μL, 3×10 copies/μL, 3 copies/μL, and negative control

Specificity testing was carried out using nucleic acids from CRSV, CPMV, CaMV, CLV, and CarMV samples. The results demonstrated specific amplification exclusively in CERV samples, with no cross-reactivity observed among the related species (Figure 3). These findings confirm the high specificity of the developed detection method.



1: CERV; others: CRSV, CPMV, CaMV, CLV, and CarMV

Figure 3. *Specificity analysis results.*

4. Discussion

CERV, a member of the genus *Caulimovirus* (family Caulimoviridae), ranks as the second most economically significant viral pathogen affecting global carnation production. The isometric viral particles (47 nm in diameter) encapsidate a double-stranded circular DNA genome of 7,932 bp [15]. Field observations reveal systematic necrotic patterning, including chlorotic rings, linear streaks, and coalescing lesions

on foliar tissues, accompanied by reduction in lateral shoot formation and flower yield loss across commercial cultivars. Notably, floral development exhibits delays with concomitant quality deterioration, particularly in petal pigmentation and structural integrity [16]. Epidemiological studies confirm semi-persistent transmission via the green peach aphid (*Myzus persicae*) [17], with secondary dissemination facilitated through global trade of asymptomatic cuttings, vegetative propagation of infected stock plants, and contaminated horticultural tools.

Current diagnostic protocols include biological indexing using corresponding hosts [9], serological detection via DAS-ELISA [18], and molecular confirmation [19] through end-point PCR and so on. Among these, biological indexing is time-consuming, which takes 2 weeks or more. Serological detection is less sensitive than molecular methods. In this study, a real-time fluorescent PCR detection method for CERV was established. Species-specific primers and probes were designed based on the conserved genomic region (accession No. AJ853858.1, from 1500 to 1808). After initial screening, an optimal primer-probe combination was settled down out of two primer pairs and four probes. Subsequently, amplification procedure was optimized to establish a robust PCR protocol. Sensitivity analysis using serially diluted plasmid concentrations showed a detection limit at 3×10^3 copies/μL, similar to end-point PCR. Specificity validation against related viruses (CRSV, CPMV, CaMV, CLV, CarMV) confirmed no cross-reactivity. The developed new method significantly enhances diagnostic efficiency in port laboratories, reducing testing time from days to 7 hours. This advancement is aimed to strengthens phytosanitary surveillance capabilities, safeguarding national biosecurity against transboundary spread of CERV through horticultural trade.

The integration of this real-time PCR assay into global phytosanitary frameworks [20] holds transformative potential for harmonizing quarantine protocols with the demands of modern horticultural trade [21]. The assay could also serve as a frontline diagnostic tool at ports of entry, enabling inspectors to screen imported carnation cuttings or tissue cultures within several hours—a critical improvement over end-point PCR or serological methods. Its closed-tube design and automation compatibility would streamline workflows in high-volume testing facilities, reducing bottlenecks in perishable commodity inspections. Embedding this assay into certification schemes for “clean” planting material would further prevent asymptomatic spread [22], addressing a key weakness in current visual inspection regimes.

5. Conclusion

CERV (genus Caulimovirus, family Caulimoviridae) poses significant threats to sustainable carnation production, which requires robust diagnostic tools to mitigate transboundary transmission via global horticultural trade. This study developed a TaqMan-based real-time PCR assay targeting a conserved genomic region (GenBank accession AJ853858.1, positions 1500–1808). Through systematic evaluation of two primer pairs and four probes, an optimized detection system achieved a sensitivity limit of 3×10^3 copies/μL, demonstrating equivalent sensitivity to conventional PCR. Specificity testing confirmed no cross-reactivity with four taxonomically related viruses: Carnation ringspot virus (genus Dianthovirus), Cowpea mosaic virus (genus Comovirus), Cauliflower mosaic virus (genus Caulimovirus), and Carnation latent virus (genus Carlavirus). Thermal cycling procedure was optimized as follows: 95 ℃ for 30 s (initial denaturation), followed by 40 cycles of 95 ℃ for 10 s, 56 ℃ for 15 s, and 60 ℃ for 20 s. This advancement enhances port inspection efficiency by reducing diagnostic time without compromising accuracy, thereby supporting global phytosanitary efforts to intercept asymptomatically infected planting materials in trade.

Abbreviations

CERV	*Carnation Etched Ring Virus*
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
CRSV	*Carnation Ringspot Virus*
CPMV	*Cowpea Mosaic Virus*
CaMV	*Cauliflower Mosaic Virus*
CLV	*Carnation Latent Virus*

Author Contributions

Wang Jiaying: Methodology
Cui Junxia: Supervision
Sun Jiayu: Data curation
Shen Jianguo: Investigation
Yu Cui: Resources
Chen Xianfeng: Conceptualization

Funding

This study received support from the Ningbo "Science and Technology Innovation Yongjiang 2035" Key Technology Project (2024Z275) and the Research Foundation of the General Administration of Customs of the People’s Republic of China (2023HK048).

Conflicts of Interest

The authors declare no conflicts of interest.

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