Bulgarian Journal of Agricultural Science, 32 (No 3) 2026, 667–673

In silico re-assessment of RT-qPCR assays for detection of tomato brown rugose fruit virus

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Abstract

Haghi, M. & Erilmez, S. (2026). In silico re-assessment of RT-qPCR assays for detection of tomato brown rugose fruit virus. *Bulg. J. Agric. Sci., 32*(3), 667–673

Tomato brown rugose fruit virus (ToBRFV) is an emerging and rapidly spreading RNA virus that infects tomato and pepper, with tomato as the primary host. In this study, we aimed to evaluate the variability in primer/probe binding regions of the Real-time RT-PCR (RT-qPCR) assays recommended by the European and Mediterranean Plant Protection Organization (EPPO) Standard on Diagnostics PM 7/146 (2). By analyzing ToBRFV sequences from the NCBI Virus database, variations in primer/probe binding sites were identified, which may impact detection accuracy. The exact match rates for the assays were as follows: ISF-ISHI-Veg CaTa 28: 76.58%, CSP1325: 93.2%, Menzel and Winter: 93.6%, and Bernabé-Orts: 95.63%. This study systematically assesses the inclusivity of these assays, highlighting specific mismatches that could lead to false-negative results. These findings emphasize the importance of continuously validating RT-qPCR methods to ensure accurate detection across diverse viral strains. Given the observed genetic variability, region-specific sequencing and *in silico* analysis are crucial to improving the robustness and reliability of RT-qPCR assays for ToBRFV.

Keywords: ToBRFV; RT-qPCR; EPPO PM 7/146 (2); sequence tracer; entropy calculator

Introduction

Tomato brown rugose fruit virus (ToBRFV) is an emerging and rapidly spreading RNA virus that infects tomato and pepper crops, with tomato as the primary host. The virus causes severe losses and threatens tomato production worldwide (Skelton et al., 2023).

ToBRFV has been included in EU regulation (Commission Implementing Decision EU 2019/1615) since November 2019, and is listed on the European and Mediterranean Plant Protection Organization (EPPO) alert list due to the harmful effects of the virus on the tomato and pepper crops (Rizzo et al., 2021).

Different techniques have been employed to detect the ToBRFV in seeds, leaves, or petioles, including serological and nucleic acid-based detection techniques. Serological methods such as enzyme-linked immunosorbent assay (ELISA) and Western-blot are currently used. However, these methods lack species-specificity and cannot be used to distinguish between ToBRFV and closely related tobamoviruses such as TMV and ToMV. This is due to the high conservation of the tobamovirus CP, resulting in antibody cross-reactivity between tobamovirus species (Panno et al., 2019). Nucleic acid-based techniques with specific primers such as reverse transcription polymerase chain reaction (RT-PCR), real-time RT-PCR, and reverse transcription loop-mediated isothermal amplification (Almeida et al., 2018; Rizzo et al., 2021).

The 6,392 nt long genome of ToBRFV is composed of four reading frames (ORFs), replication proteins, a movement protein (MP), a coat protein (CP), and 5′ and 3′ untranslated regions (Luria et al., 2017). The EPPO (European and Mediterranean Plant Protection Organization) Standard

on Diagnostics PM 7/146 (2) has recommended validated RT-qPCR assays, in which MP and CP regions are selected as the target regions (EPPO Bulletin., 2022). The validated RT-qPCR assay should be able to detect the various genetic variants of ToBRFV with high specificity and sensitivity. However, the genetic diversity of ToBRFV is poorly understood, and the variety of the ToBRFV population has only been the subject of a few studies so far (Abrahamian et al., 2022). Therefore, the inclusivity of the RT-qPCR assay is a crucial quality parameter that needs to be regularly checked through *in silico* re-assessment of the primers and the probe binding sites on the publicly available sequences to ensure precise detection of different variants of ToBRFV.

In this study, we aimed to evaluate the primer/probe binding region variability of thereal-time RT-PCR assays recommended by EPPO PM 7/146 (2). The CaTa28 and CSP1325 primers and probes from (ISF, 2020), the primers and probe from (Menzel and Winter, 2021) and primer/probe set designed by Bernabé-Orts et al. (2021).

Materials and Methods

Viral sequences data set

The whole genome sequences, including reference genomes Table 1) and partial sequences (> 6000nt) of ToBRFV were retrieved from the NCBI virus database (https://www.ncbi.nlm.nih.gov/labs/virus/vssi/) and saved as a FASTA file.

Large-scale multiple sequence alignment

MAFFT (Multiple Alignment using Fast Fourier Transform) alignment was performed on the FASTA file using an online tool (https://mafft.cbrc.jp/align ment/server/) as previously described (Katoh et al., 2019). The alignment result was downloaded as a FASTA file and viewed by AliView software.

Alignment processing

For each primer/probe set, the concatenated alignment regions starting from the forward primer first nucleotide and ending nucleotide of the reverse primer were selected and saved as a FASTA file by using AliVew software for further analysis.

Evaluation of sequence variability in primer-probe binding regions

For positional nucleotide numerical summary (PNNS), the FASTA file including primer-probe-primer was submitted to the alignment explore PNNS calculator tool (http://entropy.szu.cz:8080/EntropyCalcWeb/pnns). Following the PNNS analysis, entropy calculation (http://entropy.szu.cz:8080/EntropyCalcWeb/) was performed (Nagy et al., 2015). The entropy plot illustrating sequence variability in each position of informative sequences was created using BioEdit software (Hall, 1999).

Sequence stratification and RT-qPCR assay inclusivity

For sequence stratification, the primer-probe-primer alignments FASTA file was submitted to the Sequence Tracer option of the Alignment Explorer (http://entropy.szu.cz:8080/EntropyCalcWeb). The data was then classified as follows: informative sequences, "outgroup1" as uncertain sequences, "outgroup2" as short sequences and "excluded" as missing sequences.

Table 1. Mismatch in the primer and probe sequences of different RT-qPCR assays recommended by the EPPO PM 7/146 (2)

RT-qPCR method	Primer/ Probe	Sequence (5′–3′)	Start position	Ref. Sequence	Target gene	Total number of analysed sequences	Number of sequences with exact match	Number of informative sequences
CaTa 28	Forward	GGTGGTGTCAGTGTCTGTTT	5163	NC_028478.1	*MP*	251	193 (76,58%)	251
	Probe	AGAGAATGGAGAGAGCGGACGAGG	5191					
	Reverse	GCGTCCTTGGTAGTGATGTT	5283					
CSP1325	Forwaed	CATTTGAAAGTGCATCCG GTT T	6144	NC_028478.1	*CP*	251	235 (93,2%)	251
	Probe	ATGGTCCTCTGCACCTGCATCTTGAGA	6169					
	Reverse	GTACCACGTGTGTTTGCAGACA	6223					
Menzel and Winter (2021)	Forward	CAATCAGAGCACATTTGAAAGTGCA	6133	NC_028478	*CP*	251	236 (93,6%)	251
	Probe	ACAATGGTCCTCTGCACCTG	6166					
	Reverse	CAGACACAATCTGTTATTTAAGCATC	6203					
Bernabé-Orts et al. (2021)	Forward	CAGATGTGTCGTTGGTCAGAT	3213	MK648157	*RdRP*	251	241(95,6%)	251
	Probe	CGTAGCTTTGTCAAGGCATACCCAAA	3283					
	Reverse	CATCACTACGGTGTAATACTTC	3313					

Source: Authors' own elaboration

Results

The whole-genome sequences of ToBRFV were obtained from the NCBI Virus database and analysed for sequence similarity against different sets of primers and probes recommended by the EPPO PM 7/146(2) RT-qPCR methods (Table 1). Following multiple sequence alignment with MAFFT, variations in the sequences were found and displayed using Sequence Tracer and the Entropy Calculator. Based on nucleotide variations, different groups were identified from the collected results (Figure 1). A threshold value of ≥ 0.5% was established, and only sequences exhibiting variation frequency over this limit were taken into consideration (Khan and Cheung, 2020).

CaTa 28 and CSP1325 primers and probe sets of duplex RT-qPCR recommended by ISF-ISHI-Veg (ISF, 2020) showed an exact match with 193 (76.58%) and 235 (93.2%) genome sequences, respectively. However, a major single nucleotide mutation of the CaTa 28 probe binding region at the 5192th position displays a mismatch (G5192A) with 54 sequences (21.4%). Mismatches were also detected in CSP1325 forward primer (C6160T), probe (T6184A) and reverse primer (C6229T) binding regions with frequencies of 1.190%, 0.794% and 0.794%, respectively.

Primer and probe set by Menzel and Winter (2021) showed an exact match with 236 (93.6%) sequences. However, a mismatch (T6184A) in the probe binding region and another (T6220C) in the reverse binding region were detected with a frequency of 0.79% for both regions.

KT383474 was used as a reference sequence to design primers and the probe for the RT-qPCR method designed by Panno et al, (2019). However, the MAFFT alignment results indicated a single mutation at the 5607th nucleotide position. Moreover, the NC_028478 sequence mentioned in PM 7/146

A) ISF/ CATA

Group Number	Variant Count	Frequency %	10 20 30 40 50 130 14
			GGTGGTGTCAGTGTCTGTTT---AGAGAATGGAGAGAGCGGACGAGG---AACATCACTACCAAGGACGC
1	193	76,587	~~~ ~~~
2	54	21,429	a ~~~ ~~~
3	1	0,397	t a ~~~ ~~~
4	1	0,397	~~~ ~~~ t..
5	1	0,397	t ~~~ ~~~
6	1	0,397	a ~~~ a ~~~
7	1	0,397	a ~~~ ~~~

B) CSP 1325

Group Number	Variant Count	Frequency %	10 20 30 40 50 80 90 100
			CATTTGAAAGTGCATCCGGTTT---ATGGTCCTCTGCACCTGCATCTTGAGA---TGTCTGCAAACACACGTGGTAC
1	235	93,254	~~~ ~~~
2	3	1,190	t ~~~ ~~~
3	2	0,794	~~~ a ~~~
4	2	0,794	~~~ ~~~ t
5	1	0,397	a ~~~ ~~~
6	1	0,397	t ~~~ ~~~
7	1	0,397	~~~ a ~~~
8	1	0,397	c ~~~ ~~~
9	1	0,397	a ~~~ ~~~
10	1	0,397	t ~~~ ~~~
11	1	0,397	~~~ c ~~~ t
12	1	0,397	a ~~~ ~~~ t
13	1	0,397	g ~~~ ~~~
outgroup2	1	0,397	

Fig. 1. Alignment stratification of the primers and probe binding regions of the 251 ToBRFV sequences. The figure shows the group number, variant count and the frequency of the variants. The identical nucleotides are represented by dots in the sequences. "outgroup 2" represents the short sequences. The threshold value was ≥ 0.5 %.

Source: Authors' own elaboration

C) Menzel & Winter

Group Number	Variant Count	Frequency %	10 20 40 50 80 90
			....|....|....|....|....| .|....|....|....|... ....|....|....|....|....|.
			CAATCAGAGCACATTTGAAAGTGCA---ACAATGGTCCTCTGCACCTG---GATGCTTAAATAACAGATTGTGTCTG
1	236	93,651	.....~~~.....~~~.....
2	2	0,794	.....~~~.....a.~~~.....
3	2	0,794	.....~~~.....~~~.....c.....
4	1	0,397	.....t.....~~~.....~~~.....
5	1	0,397	.....~~~.....~~~.....g.....
6	1	0,397	.....~~~.....a~~~.....
7	1	0,397	.....c.....~~~.....~~~.....
8	1	0,397	.....a.....~~~.....~~~.....
9	1	0,397	.....a....~~~.....~~~.....
10	1	0,397	.....t.~~~.....~~~.....
11	1	0,397	.....~~~.....c.....~~~.....
12	1	0,397	.....a..~~~.....~~~.....
13	1	0,397	.....~~~.....~~~.g.....
14	1	0,397	.....g~~~.....~~~.....
outgroup2	1	0,397	

D) Bernabé-Orts et al

Group Number	Variant Count	Frequency %	10 20 80 90 110 120
			....|....|....|....|. ....|....|....|....|....|. ....|....|....|....|..
			CAGATGTGTCGTTGGTCAGAT---CGTAGCTTTGTCAAGGCATACCCAAA---GAAGTATTACACCGTAGTGATG
1	241	95,635	.....~~~.....~~~.....
2	8	3,175	....c.....~~~.....~~~.....
3	2	0,794	.....~~~.....~~~.....t.....
4	1	0,397	.....~~~t.....~~~.....

Fig. 1. Continued

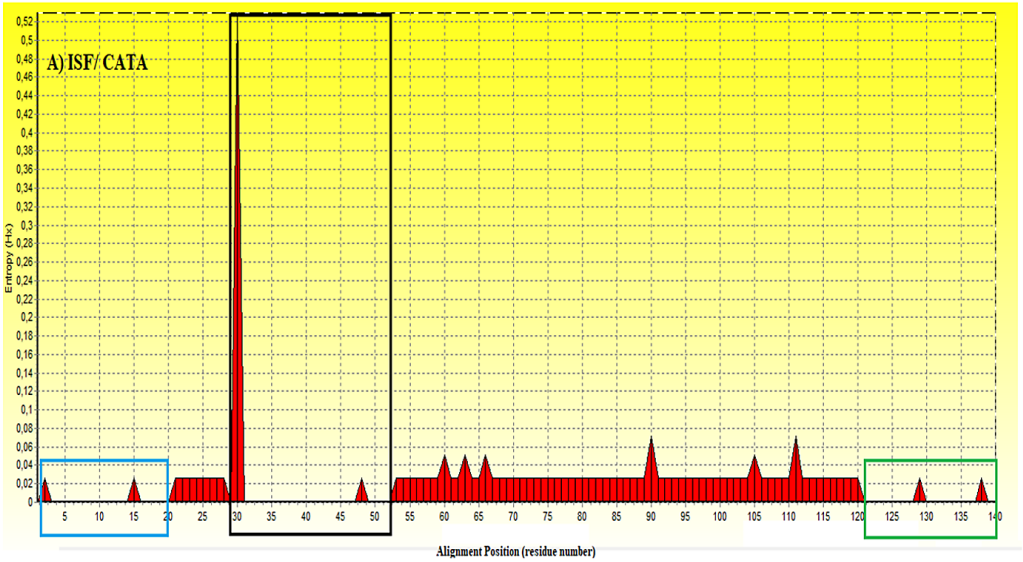


Fig. 2. The entropy plot

The nucleotide variability of each position of RT-qPCR amplicons is illustrated. The related binding regions of the forward primer (blue), probe (black) and reverse (green) are highlighted in the amplicons

Source: Authors' own elaboration

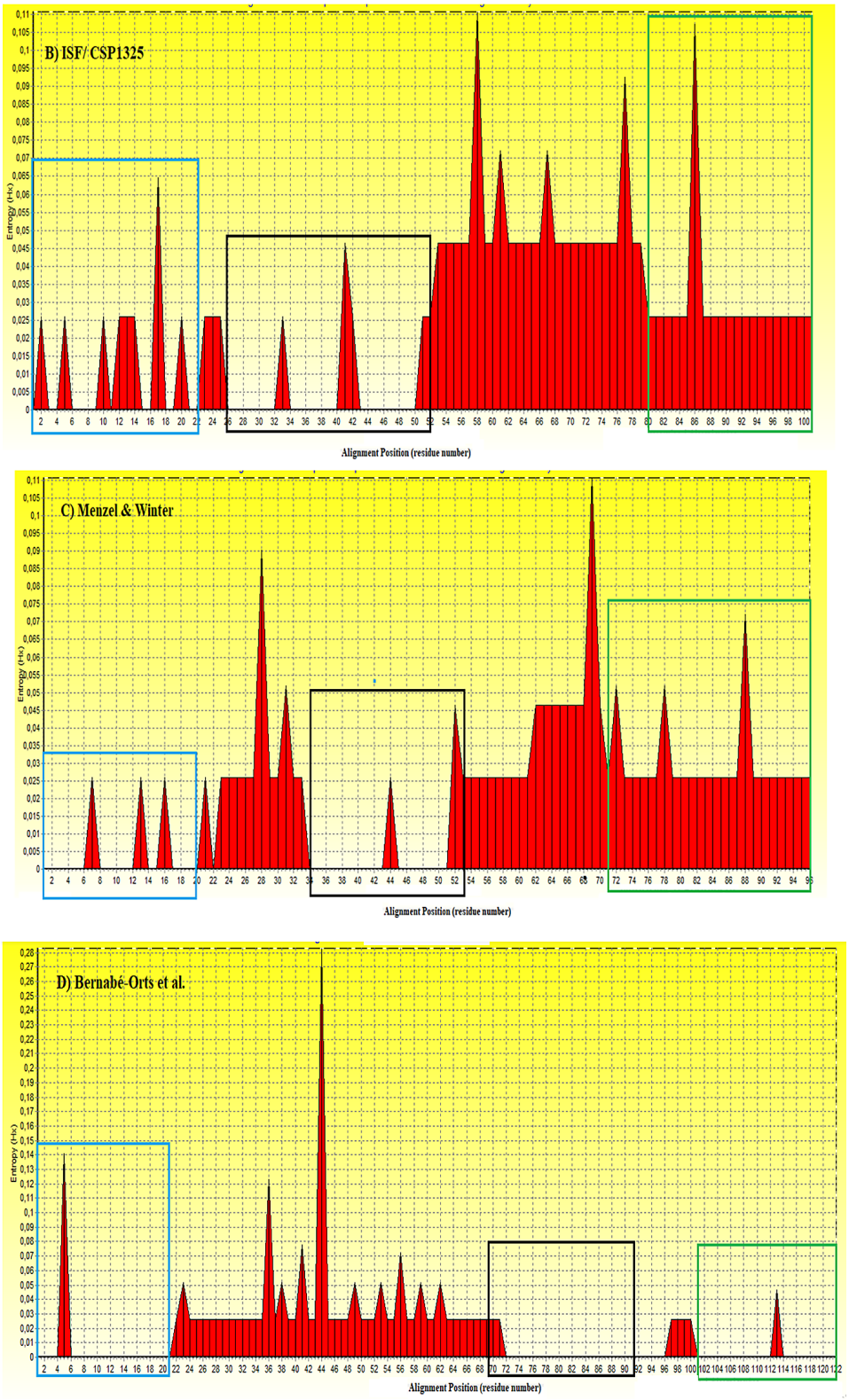


Fig. 2. Continued

(2) also contains the same mutation. Between 2014 and 2019 only eight whole genome sequences were deposited on the NCBI database. Six sequences showed the same mutation. In the mentioned study, the following sequences were used for the alignment analysis: MK133093, MK133095, KX619418, KT383474, except MK133095, others have the same mutation. Among the 251 sequences, only 31 (12.3%) sequences have this mutation, leading to a mismatch (A5607G) in the reverse primer binding site. Therefore, this method was not considered for further analysis.

Considering the RT-qPCR method developed by Bernabé-Orts et al. (2021), 241 (95.63%) sequences showed an exact match with primers and the probe binding regions. A mismatch (T3217C) in the forward primer region and a mismatch (C3325T) in the reverse primer region were detected with frequencies of 3,175% and 0,794%, respectively. The entropy plot illustrated the nucleotide variability of each position of the RT-qPCR amplicons (Figure 2).

Discussion

The genome diversity of the ToBRFV is limited. However, in the ToBRF genome, *CP* is the most conserved gene compared to *RdRp* and *MP* genes (Abrahamian et al., 2022; Lyu et al., 2023).

The stratification of ToBRFV sequences with the primers and the probe sequences using RT-qPCR methods revealed mismatches in the primers and the probe binding regions.

Mismatches can occur at both 3′ and 5′ ends. A single-nucleotide mismatch at the 3’ end can adversely affect Taq polymerase and consequently the PCR reaction (Lefever et al., 2013).

Furthermore, mismatches in the probe may have a negative impact on the PCR amplification. Even a single mismatch can lead to false-negative results by inhibiting the binding of the probe to the target region PCR amplification (Brault et al., 2012).

Considering the ISF RT-qPCR method, stratification of the sequences with concatenated CaTa 28 primers and probe showed a major mismatch (G5192A) in the probe binding region of 54 sequences, which were of Netherlands (47 sequences) and Belgium (7 sequences) origins.

Three sequences MN882049.1, MN882050.1 and OM515233.1 from the Netherlands showed a mismatch (C6160T) with CSP1325 forward primer, and two Belgian sequences MZ945419 and MZ945420 showed a mismatch (C6229T) with CSP1325 reverse primer. A mismatch (T6184A) was also detected in the probe binding region of OQ633216.1 and OQ674194.1 Belgian isolates.

Belgian isolates MZ945419.1 and MZ945420.1 had a mismatch (T6184A) at the 3′ of the probe designed by Menzel and Winter (2021). Two other Belgian isolates OR760198 and OR760199 showed a mismatch (T6220C) with the reverse primer binding region.

A Greek isolate (OQ190155.1) and seven isolates from the Netherlands (MN882030, MN882031, MN882032, MN882033, MW314092, MW314093, MW314094) showed a mismatch (T3217C) with the forward primer binding region developed by Bernabé-Orts et al., 2021). A mismatch (C3325T) in the reverse primer binding region of American isolates OM892676 and OM892678 was detected.

Conclusion

This study highlights the importance of *in silico* re-assessment of RT-qPCR assays for the detection of Tomato Brown Rugose Fruit Virus (ToBRFV) by analysing the binding accuracy of the various primers and probes suggested by the EPPO PM 7/146 (2), revealing variability in primer and probe binding regions that may impact the accuracy of RT-qPCR methods. The method developed by Bernabé-Orts et al. (2021) showed the highest match with the analysed sequences among the other methods. It is advisable for researchers across different regions to conduct extensive viral gene sequencing studies. Such efforts will help identify region-specific mutations in primer and probe binding sites, ensuring that RT-qPCR assays maintain high sensitivity and specificity across different ToBRFV variants. By applying these findings, RT-qPCR methods can be enhanced to minimize false-negative results, strengthen surveillance, and improve ToBRFV management strategies**.**

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical statement

This is a bioinformatics study, and no ethical approval is required.

Data availability

All data generated or analysed during this study are included in this published article.

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Received: **January, 07, 2025**; *Approved*: **March, 10, 2025**; *Published*: **June, 2026**