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Research

Pilot for Harmonization of Diagnostic Protocols for Tomato Brown Rugose Fruit Virus (ToBRFV) in Tomato and Pepper Seeds

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Abstract

Global seed trade is subject to various national, regional, and international regulations to prevent the introduction and spread of harmful seed-borne and seed-transmitted pathogens. When the plant health regulatory agencies of trading partners employ different diagnostic protocols for the same pathogen, contradictory test results may require additional testing that can cause delays in trade. Establishing equivalency of diagnostic protocols may expedite trade by adding confidence to diagnostic test results. The member countries of the North American Plant Protection Organization (NAPPO) conducted a project evaluating several diagnostic protocols for a seed-transmitted virus, tomato brown rugose fruit virus (ToBRFV), an emerging pathogen that has severely affected tomato and pepper fruit and seed production and trade globally. The objective of the study was to find protocols that could be harmonized among NAPPO member countries, thereby avoiding retesting of samples at different border points. The project was a collaboration between academia, industry, trade organizations, and national plant protection organizations (NPPOs). Three end-point PCR and two real-time PCR protocols were evaluated via a ring test. Nine laboratories from Canada, the United States, and Mexico participated in the ring test, which generated 3,680 data points from analytical, diagnostic, and calibrator samples. Four out of five diagnostic protocols were found to be fully transferable, and three protocols demonstrated optimal performance for accurate, reproducible, and user-friendly detection. The results of this regional effort will simplify the detection of ToBRFV-infected seeds in NAPPO member countries and demonstrate a way to establish equivalency of testing methods between the NPPOs.

Keywords: diagnostic ring test, end-point RT-PCR, molecular diagnostics, real-time RT-PCR, ToBRFV

The calculated economic losses to global agriculture from plant virus damage are approximately $30 billion per year (Nicaise 2014). Tobamoviruses are some of the most damaging plant viruses to vegetable and ornamental crops around the world due to stability of the virions to remain infectious for many years under various environmental conditions. Tobamoviruses are easily mechanically transmitted; several are also seed transmitted, which facilitates their spread around the world via global seed trade (Dombrovsky and Smith 2017). Significant efforts to reduce this spread have led to the development of virus-resistant tomato and pepper varieties, sanitation options, and establishment of quarantine and phytosanitary restrictions by plant protection organizations around the world.

In 2015, a new tobamovirus, named tomato brown rugose fruit virus (ToBRFV), was described on greenhouse tomatoes in the Jordan Valley (Salem et al. 2016). The symptoms induced included mild leaf mosaic and deformation and strong brown rugose areas on fruits that reduced their marketability. Further molecular analyses and bioassays confirmed its taxonomy (Schoch et al. 2020). Currently, this emergent virus has been identified in at least 25 countries on four continents (Asia, Europe, North America, and Africa) (Salem et al. 2023), including all three North American Plant Protection Organization (NAPPO) countries: Canada (Sarkes et al. 2020), the United States (Dey et al. 2021; Ling et al. 2019), and Mexico (Cambrón-Crisantos et al. 2019). Just recently, the virus was confirmed in South Australia (PIRSA 2024). In 2023, the International Committee on Taxonomy of Viruses (ITCV) proposed a new binomial name for this virus, *Tobamovirus fructirugosum*, genus *Tobamovirus*, family *Virgaviridae* ICTV 2024.

ToBRFV is considered a serious threat to its main natural hosts, tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*), as it breaks the existing tobamovirus resistance (Salem et al. 2023). Therefore, ToBRFV is regulated in many countries, with trade of tomato and pepper seeds experiencing considerable regulatory restrictions. The European and Mediterranean Plant Protection Organization (EPPO) Secretariat added ToBRFV to their alert list (EPPO 2020), and the European Commission implemented a strategy to prevent the introduction and spread of ToBRFV to EU countries (EPPO 2020). The United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) issued Federal Orders requiring a declaration of pest freedom prior to the importation of tomato and pepper products (USDA 2019b, 2020, 2024). The introduction and further spread of ToBRFV in North America represents a significant threat because solanaceous seeds, seedlings, and fruit are heavily traded among NAPPO member countries (NAPPO Expert Group 2022). Considering the importance of this pathogen in North America, the NAPPO Executive Committee approved a project to compare ToBRFV diagnostic protocols used by the NAPPO members for seed testing and recommend one or more of them for use by the region's national plant protection organizations (NPPOs). Using different diagnostic methods could produce differing results; thus, harmonization of diagnostic protocols for ToBRFV in North America would eliminate delays and reduce the costs of retesting seed lots at different border points, thereby facilitating trade.

Multiple methods for the diagnosis and detection of ToBRFV have been reported, including electron microscopy to observe viral particles (Luria et al. 2017; Mahillon et al. 2022), serological techniques with polyclonal antibodies to immunodetect the viral coat protein (Mrkvová et al. 2022), various reverse transcription-polymerase chain reaction (RT-PCR) methods for the detection of different regions of virus genomic RNA (Caruso et al. 2022), droplet digital PCR assays (Vargas-Hernández et al. 2022), and next-generation sequencing (Abrahamian et al. 2022). The International Seed Federation/International Seed Health Initiative for Vegetables (ISF ISHI-Veg) developed a real-time RT-PCR targeting two genomic regions, the coat protein, and movement protein-encoding RNAs (ISF ISHI-Veg 2024) that is widely used by industry and other testing entities around the world. Recently, the European Phytosanitary Research Coordination (Euphresco) conducted two interlaboratory comparisons of several detection methods of ToBRFV in plants (Luigi et al. 2022) and seeds (Giesbers et al. 2021) prior to development of the EPPO Standard PM7/146 (2)-Diagnostics (EPPO 2022). In the NAPPO region, additional diagnostic protocols, due to regional preferences, have been validated for use by each NPPO. It was important to conduct a similar study to evaluate the diagnostic protocols used by member countries for ToBRFV detection in tomato and pepper seeds and establish their comparability (i.e., evaluate similarities and differences in methods' performance characteristics, such as accuracy, precision, specificity, and detection limit).

NAPPO assembled a group of experts representing academia, regulatory agencies, and industry from the three NAPPO member countries to select diagnostic protocols, propose the experimental design, identify the participating laboratories, manage the logistics of distributing the ring test samples and diagnostic reagents, and collect and analyze the data obtained.

Materials and Methods

Diagnostic protocols

Five end-point and real-time RT-PCR diagnostic protocols (A, B, C, D, and E) (Table 1) employed for ToBRFV phytosanitary testing in seeds by NAPPO member countries and major trading partners were selected. Each protocol has been validated by the proposing NPPOs or industry for ToBRFV detection in seeds prior to this study. In addition, Protocol B and primers of Protocol E included in a ready-to-use kit developed by Loewe,

TABLE 1

Tomato brown rugose fruit virus diagnostic protocols evaluated in this study

Protocol ID	RT-PCR type	Target genes	Internal control	Primer/probe reference
A	One step, end-point	MP[a]	None	T. Tian, *unpublished data*
B	One step, real-time	MP, CP[b]	*nad5*[c]	ISF ISHI-Veg 2024
C	One step, real-time	MP	*nad5*	Chanda et al. 2021
D	One step, end-point	CP	None	Dey et al. 2021
E	Two steps, end-point	RdRP[d]	18S RNA	Rodríguez-Mendoza et al. 2019

[a] MP, movement protein-encoding gene.
[b] CP, coat protein-encoding gene.
[c] *nad*5, mitochondrial NADH dehydrogenase subunit 5 plant gene; 18S RNA, ribosomal 18S plant RNA. Protocol A was provided by the Canadian Food Inspection Agency, protocol B by the USDA National Seed Health System, protocols C and D by USDA, APHIS, PPQ, and protocol E by SENASICA.
[d] RdRP, RNA-dependent RNA polymerase-encoding gene.

the tomato brown rugose fruit virus-Complete One-Step Reverse transcriptase PCR Reaction Kit (Loewe Biochemica, Sauerlach, Germany), were included in the Euphresco's ToBRFV test performance studies for seeds (Giesbers et al. 2021) and plants (Luigi et al. 2022).

Laboratories

Nine laboratories from Canada, the United States, and Mexico (Table 2), representing academic institutions, regulatory agencies, and commercial entities, agreed to participate in the study and provide results.

Seeds

ToBRFV-infested tomato seeds (referred to as positive hereafter) were donated by the seed industry to the California Seed and Plant Laboratories, an ISO/IEC 17025:2017 and National Seed Health System (U.S.A.)-accredited testing laboratory, to be used as reference material in research and diagnostic testing. The California Seed and Plant Laboratories, in turn, mixed seeds from different producers to create a single lot and provided it to the NAPPO project. ToBRFV-free tomato and pepper seeds (referred to as healthy hereafter) were purchased from commercial sources and verified to be free of ToBRFV.

In vitro transcripts

In vitro transcripts for three ToBRFV target regions, namely RNA-dependent RNA polymerase, movement protein (MP), and coat protein (CP) were developed using commercial kits by the Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV, Mexico), and tomato mottle mosaic virus (ToMMV), a closely related tobamovirus, in vitro transcripts in the corresponding target regions were developed and produced by the Plant Pathogen Confirmatory Diagnostic Laboratory (PPCDL, U.S.A.). Each plasmid template and each transcript was sequenced to confirm identity and integrity. The concentration of each was quantified using an HS RNA Qubit assay and calculated as copy numbers.

TABLE 2

Laboratories participating in the North American Plant Protection Organization ring test

Name	Country	Type
Charlottetown Laboratory, Canadian Food Inspection Agency (CFIA)	Canada	Government
Ottawa Plant Laboratory, CFIA	Canada	Government
Plant Pathogen Confirmatory Diagnostic Laboratory (PPCDL), APHIS	U.S.A.	Government
Seed Science Center, Iowa State University	U.S.A.	Academia
California Seed and Plant Laboratory	U.S.A.	Private
Plant Diagnostic Center, University of Florida	U.S.A.	Academia
Laboratorio de Virología, CNRF/SENASICA	Mexico	Government
Laboratorio de Biología Molecular y Genómica Funcional, CIAD	Mexico	Academia
Laboratorio de Diagnóstico Integral Fitosanitario (LADIFIT)	Mexico	Academia

Experimental design and panel composition

A ring test, where the same set of samples (ring test panel) are tested by each participating laboratory using the selected diagnostic protocols, was designed in accordance with the internationally accepted principle for diagnostic methods validation (ISO/IEC 17025:2017 2020); Eurachem (Magnusson and Örnemark 2014); and the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use -Analytical Validation Q2(R1) (ICH 2023). The five molecular RT-PCR protocols selected were labeled as A, B, C, D, and E to minimize bias during testing (Table 1). The selected protocols included three end-point (A, D, and E) protocols and two real-time RT-PCR (B and C) protocols. Participating laboratories used their own RNA extraction protocols for seed samples; no efforts were made toward harmonization of this step. RT-PCR reagents for each protocol, including primers and probes, were purchased and provided by NAPPO to minimize the lot-to-lot variability from the manufacturing source.

A pre-test was conducted prior to the ring test to ensure that the reagents provided worked with the RNA extracted by each laboratory. This additional step allowed participating laboratory staff to familiarize themselves with the protocols while using their own RNA extraction protocol.

The ring test panel composition and schematic were designed to assess each protocol's sensitivity, specificity, and precision, as detailed in Table 3. All samples were blinded, and all controls were identified for the lab. Positive and healthy tomato seed (samples B and D, respectively) and pepper seed (sample E) were used to compare the selected protocols. The ToBRFV in vitro transcript (sample A) was used as a quality check of the result of the ToBRFV-positive tomato seed sample (sample B). ToMMV in vitro transcript (sample C) in two concentrations provided data to assess test specificity. A calibrator sample set was included to provide quality assurance for results generated using different real-time PCR instruments. Samples B, D, and E were also

TABLE 3

Ring panel composition and sample description

Sample ID	Description	Samples provided
Sample A	Tomato brown rugose fruit virus (ToBRFV) in vitro transcripts[a] in TE[b] buffer. Five samples created before shipment by serially diluting sample A 10× (A-1, A-2, A-3, A-4, and A-5).	5 (10×) dilutions in TE buffer
Sample B	ToBRFV-infested (positive) seed sample at a relatively high concentration. Extracted RNA was then 10× serially diluted in the lab with TE buffer to create a 5-point curve (B-1, B-2, B-3, B-4, and B-5).	2 samples, 1,000 seeds each, for extracting RNA
Sample C	ToMMV in vitro transcripts[a] in TE buffer; 2 dilutions (C-1, high concentration; C-2, a 1:100 dilution of C-1).	2 dilutions in TE buffer
Sample D	ToBRFV-free tomato seed sample.	1 × 1,000 seeds, to extract
Sample E	ToBRFV-free pepper seed sample.	1 × 500 seeds to extract
PPC	Positive process control, ToBRFV-infested (positive) seed.	1 × 1,000 seeds to extract
NPC tomato	Negative process control, ToBRFV-free seed sample.	1 × 1,000 seeds to extract
NPC pepper	Negative process control, ToBRFV-free seed sample.	1 × 500 seeds to extract
Calibrator	ToBRFV in vitro transcripts[a] in TE buffer. Five samples created before shipment by serially diluting sample A 10× (Calibrator-1, Calibrator-2, Calibrator-3, Calibrator-4, and Calibrator 5).	5 (10×) dilutions in TE buffer
NTC	Nontemplate control, molecular grade H_2O.	Provided by each lab

[a] A mix of three in vitro transcripts in equimolar concentrations.
[b] TE is 10-mM Tris-HCL (pH 8.0) and 0.1-mM EDTA.

used as controls to observe potential processing bias. The positive process control was labeled "PPC," and two negative process controls were labeled "NPC-tomato" or "NPC-pepper."

To achieve statistical significance of the data, nine laboratories (Table 2) with two diagnosticians per laboratory participated in the ring test. One diagnostician (diagnostician #1) extracted RNA from all known and unknown seed samples and conducted all PCR tests. The second diagnostician (diagnostician #2) performed only selected RT-PCR tests to generate adequate data points to evaluate intermediate precision. Thus, the positive tomato seed sample B was evaluated twice for each assay, once by diagnostician #1 and once by diagnostician #2. The ToBRFV-positive analytical sample A, the ToMMV analytical sample C (closely related virus), and the calibrator set were tested by one of two real-time PCR protocols per laboratory. Untested protocols for these three materials were randomized between laboratories to reduce testing while generating enough data to have >85% confidence in validation categories and >90% confidence in total results for each sample.

The passing criteria for data for each protocol to be accepted for analyses were as follows:

(i) The sample A (analytical sample) percent sensitivity across a 5-point standard curve must be higher than that for the sample B (diagnostic sample) 5-point standard curve.
(ii) The sample A percent detected at the most diluted concentration must be higher than for sample B.
(iii) Samples D and E must have a percent specificity above 95%. The sample C (ToMMV analytical sample) percent specificity must not be higher than those of samples D and E (diagnostic samples).
(iv) At least three points of the calibrator curve must produce a coefficient of determination (r^2) equal to or greater than 0.98. Otherwise, data could only contribute to final sensitivity and specificity calculations for the overall study; the data could not be used for estimating a general real-time PCR cutoff.
(v) Data meeting all four criteria and having a calibrator slope of -3.32 ± 0.2 were compiled to estimate a cycle cutoff for the real-time assays.

The criteria for individual sample reactions were as follows:

(i) False-positive and false-negative reactions did not contribute to precision analysis.
(ii) Real-time PCR true negative reactions at the end-of-cycle value of 40 were included in precision analysis.
(iii) Summary analyses did not include real-time PCR outliers attributable to amplification signal anomalies, operator error identified by ring test quality checks, operator error noted by the lab, or failed ring test quality checks.
    a. Amplification anomalies include unusually low cycle values (e.g., 12 or lower) or stunted curves. A stunted curve has the same sigmoidal shape as the other positive samples, but the curve is less than half the height of the other positive samples.
    b. Ring test quality checks included failed internal controls (false negative) and failed dilution curves. Data from failed dilution curves were applied up to the concentration at which the failure was identified (no cycle value change) unless the cycle value indicated the detection limit was reached.
(iv) Weak positives for end-point PCR negative samples were considered false positives. Weak positives for positive samples were considered true positives.

Ring test panel production verification and assembly

The ring test panel was produced and verified by the PPCDL (U.S.A.). Four seed batches were prepared: two positive (ToBRFV-infested) tomato seeds (sample B and the positive process control), one healthy tomato seed, and one healthy pepper seed. The positive batches were prepared by mixing healthy tomato seed with ground positive seed to create samples of expected titer, homogeneity, and appearance. A total of 382 individual seed samples (3 g each) were placed in vacuum-sealed envelopes and stored at −80°C.

Individual transcripts were mixed in equimolar concentration to prepare analytical samples, followed by tenfold dilutions to achieve the desired virus titer. In total, 120 composite transcript samples were prepared. Fifteen percent of each seed or in vitro transcript sample batch was tested by two diagnosticians, using each of the five protocols to evaluate homogeneity and generate reference values. Measurement uncertainty was estimated for reference values to monitor results as they were submitted for evaluation; the estimated values were used to identify any material issues and were not used as pass or fail criteria. PCR reagents, primers, and probes were purchased in bulk and aliquoted to assemble 47 reagent packs for the five protocols. Reagents were assessed before distribution.

Blind, randomized ring test panels were assembled and distributed to the participating laboratories (Table 2) by the Texas Plant Disease Diagnostic Laboratory (U.S.A.), an independent laboratory not participating in the ring testing. Three standard operating procedures (SOPs) with detailed instructions for each of the five protocols were developed using the codes assigned to the protocols and samples (each sample was labeled differently within each SOP) and provided to all participating laboratories. SOPs were designed so that all protocols, but not all samples, were tested by each participating laboratory.

Diagnostic samples were tested in triplicate to capture variation resulting from sample amplification for each protocol in each laboratory. The nontemplate control, the PPC, and the calibrator were tested in duplicate. NPCs (tomato and pepper) were tested in triplicate.

Ring test panel distribution and results reporting

The ring test panels were distributed to participating laboratories according to the import regulations of each country. Temperature was monitored during shipping to ensure stability of the material.

Upon completion of testing, each laboratory entered test results into the APHIS Laboratory Portal, a USDA portal designed for proficiency testing and managed by the National Animal Health Laboratory Network (NAHLN). The module designed by NAHLN for the ring test captured unique data entries, provided confidentiality, and allowed for storage of all pictures and files in either portable document format (.pdf) or Microsoft Word (.doc) format and common graphical formats (e.g., .jpg and .png). Data entry included laboratory equipment types and chemistries, and all data were compiled and stored by the portal module in a uniform .csv file. The module was designed to prevent varied data entry formats.

Results were handled using chain-of-custody procedures. In other words, each event involving data organization for analysis was done on a new Excel worksheet, and all formulas were traceable to the original data.

Data analysis

All data were evaluated by cycle value (Cq) (real-time PCR) or percent correct (end-point PCR). Real-time RT-PCR results were also evaluated by percent coefficient of variation (CV) (standard

deviation divided by the mean; also known as relative standard deviation) to make comparisons between different concentrations and conditions. Comparisons were also made between amplification curves using the correlation coefficient (r^2) and linearity (slope and cutoff determination, Cq = slope × log copies – Cq_0). A passing copy number recovery percentage of 50% or greater for the two highest calibrator concentrations indicated the laboratory's data could be used to establish a cutoff.

Mean comparison tests (Dunnett's T3) were performed at an alpha level of 0.05 to compare the mean limit of detection (LOD) values of all samples for all protocols tested. The statistical differences were represented using a heat map (GraphPad, Prism v9.0), indicating the observed differences in the red-to-blue scale color.

Results

Data from all nine participating laboratories were used to make comparisons among the five evaluated diagnostic protocols. Data from six laboratories were used to estimate a sample cutoff of 34 cycles for real-time RT-PCR.

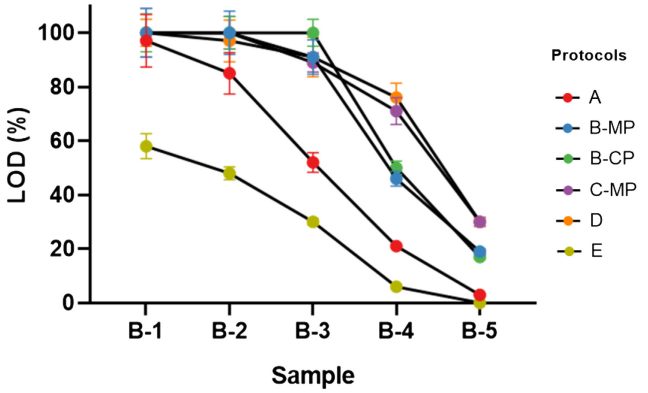


FIGURE 1
Limit of detection (LOD) of samples B-1, B-2, B-3, B-4, and B-5 (5-point serial dilution) with each tested protocol. MP, movement protein-encoding gene; CP, coat protein-encoding gene.

LOD

In this study, LOD is defined as observed true positives divided by the number of expected true positives at a specific concentration. Percentages ranged from 0% for negative samples (no ToBRFV detected) to 100% (all reactions had detectable ToBRFV at the specified concentration). LOD values for all protocols using positive seeds (sample B) and mean comparison tests for the different concentrations for each protocol are shown in Figures 1 and 2 and Supplementary Table S1. Protocols B, C, and D yielded the highest LOD values for all concentrations tested. Statistical analyses demonstrated significant differences when mean comparison was performed employing Welch's analysis of variance test and a Dunnett's T3 test, confirming that Protocols B, C, and D provided reproducible results among participating laboratories (Fig. 3)

Sensitivity

In this study, sensitivity is defined as the percentage of positive results across a dilution series. The calculation is discrete from LOD, the percent positive at a specific dilution point, to form a larger view of assay performance. For example, as shown in Figures 1 and 2, Protocol D sample B-5 had a higher percent positive rate (30.3%) for LOD at a specific dilution than Protocol B-MP (18.5%). However, Table 4 and Figure 4 show that Protocol B-MP sample B had a higher percent positive rate (90.3%) than Protocol D (78.8%) across the entire range of dilutions, from samples B-1 to B-5.

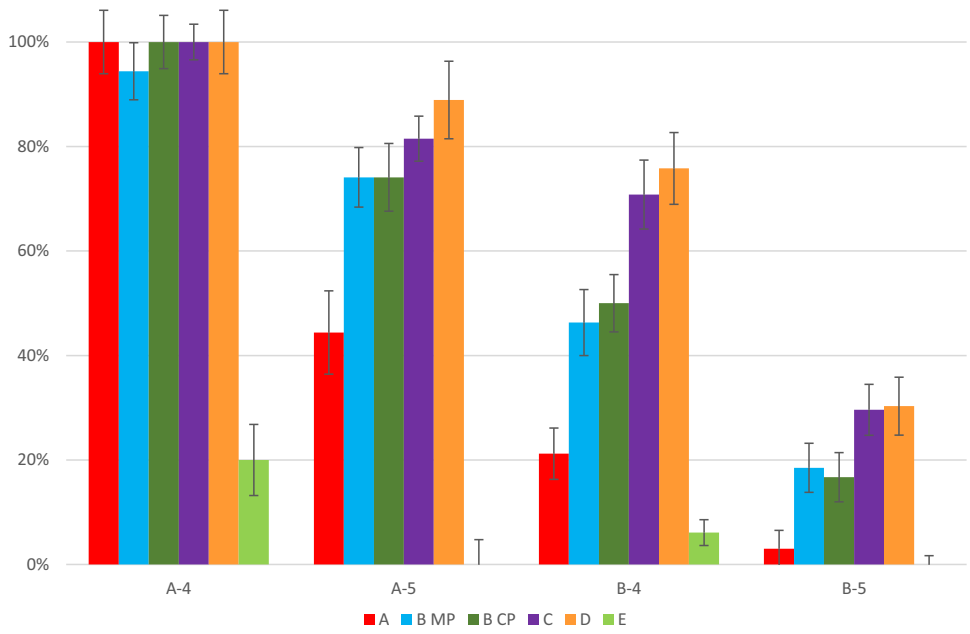
Specificity

Specificity is defined as the percentage of negative results across the pool of a ToBRFV-negative sample's results. Protocol B's specificity for the MP primer and probe set was significantly different from the other protocols for sample E (77.8 ± 3.6). The other protocols had a specificity above 95% (Table 5) for sample E.

Precision

Precision is the assessment of variability between test results for real-time Protocols B and C. Three precision parameters were evaluated: (i) repeatability, which assesses replicates in the same test; (ii) intermediate precision, which assesses variability

FIGURE 2
Percent limit of detection by protocol for the two lowest concentrations of sample A and sample B. Error bars are 95% confidence intervals. MP, movement protein-encoding gene; CP, coat protein-encoding gene.



between laboratory technicians; and (iii) reproducibility, which combines the results of all laboratories (Table 6). Repeatability ranges were less than 2% CV, intermediate precision ranges were less than 5%, and reproducibility ranges were below 10% (Table 6). These percent CV values were employed as a pass or fail criterion for the ring test, respective to the precision parameters. Precision is detailed in Table 2 of the Supporting Information section.

Linearity and accuracy

Variation among instruments used by participating laboratories was determined using a 1/10 serially diluted calibration curve. Values for at least three dilution points were used to establish the curve and obtain a coefficient of determination (r^2), which measures how well the calibration points fit the expected curve for each instrument used. Values for the nine evaluated laboratories were >0.99 (passing criteria number 4) (Table 7).

Accuracy is the slope of the calibration curve and sample cutoff as a measure of the y-intercept. The values for six participating laboratories shown in Table 7 ranged from a slope of −3.18 to −3.48. Because calibrator samples consisted of transcripts, cycle values were back-calculated to copies (Supplementary Tables S3 and S5) to verify that amplification kinetics and percent yields were correct. For example, one lab produced a standard curve with the calibrator tested using Protocol C that ranged in cycle value from 14.22 to 27.56 (5 points, slope = −3.33, intercept = 40.27). Based on the standard curve, the total copies for the highest concentration (Calibrator-1) was 6.60E + 07. The estimated copies for this calibrator was 6.66E + 07 (Supplementary Table S3). Therefore, the calculated yield was 99% correct (Supplementary Table S5, Curve 3).

Positive sample cutoff determination

Calculation of the real-time PCR assay positive sample cutoff was done using a calibrator curve to subtract one log copy from the y-intercept (Cq_0 – 3.32). A common cutoff of 34.04 ± 1.42 C_q was estimated using amplification kinetic data from six

TABLE 4

The sensitivity of positive analytical (A) and diagnostic (B) samples[a]

Sample	Protocol	Sensitivity	CI	*n*	Protocol	Sensitivity	CI	*n*
A-1	A	100.0%	3.9%	18	C	100.0%	7.1%	27
A-2	A	100.0%	5.1%	18	C	100.0%	6.4%	27
A-3	A	100.0%	5.7%	18	C	100.0%	4.4%	27
A-4	A	100.0%	6.1%	18	C	100.0%	3.4%	27
A-5	A	88.9%	8.0%	18	C	99.3%	4.3%	27
B-1	A	97.0%	10.4%	33	C	100.0%	8.7%	31
B-2	A	90.9%	9.1%	33	C	100.0%	6.0%	32
B-3	A	77.8%	6.9%	33	C	98.8%	4.4%	33
B-4	A	63.6%	4.9%	33	C	96.7%	6.6%	29
B-5	A	51.5%	3.5%	33	C	92.8%	4.9%	33
A-1	B-MP	100.0%	5.4%	25	B-CP	100.0%	7.8%	25
A-2	B-MP	100.0%	5.4%	27	B-CP	100.0%	7.2%	27
A-3	B-MP	100.0%	5.0%	27	B-CP	100.0%	5.9%	27
A-4	B-MP	99.7%	5.5%	26	B-CP	100.0%	5.1%	26
A-5	B-MP	98.6%	5.7%	26	B-CP	98.9%	6.5%	26
B-1	B-MP	100.0%	9.2%	42	B-CP	100.0%	6.6%	42
B-2	B-MP	100.0%	7.5%	41	B-CP	100.0%	5.7%	41
B-3	B-MP	98.9%	6.6%	42	B-CP	100.0%	5.3%	42
B-4	B-MP	95.2%	6.3%	42	B-CP	96.8%	5.5%	42
B-5	B-MP	90.3%	4.7%	42	B-CP	92.1%	4.7%	42
A-1	D	100.0%	3.9%	18	E	100.0%	3.9%	18
A-2	D	100.0%	5.1%	18	E	94.4%	11.1%	18
A-3	D	100.0%	5.7%	18	E	79.6%	9.0%	18
A-4	D	100.0%	6.1%	18	E	66.7%	6.8%	15
A-5	D	97.8%	7.4%	18	E	52.9%	4.8%	18
B-1	D	100.0%	4.9%	33	E	57.6%	7.6%	33
B-2	D	98.5%	7.6%	33	E	53.0%	5.3%	33
B-3	D	96.0%	7.6%	33	E	45.5%	3.8%	33
B-4	D	90.9%	6.9%	33	E	35.6%	2.5%	33
B-5	D	78.8%	5.5%	33	E	28.5%	1.7%	33

[a] Because Protocol B is a duplex real-time PCR assay, each target (movement and coat protein) was assessed. CI = 95% confidence interval; *n* = number of points evaluated. MP, movement protein-encoding gene. CP, coat protein-encoding gene.

TABLE 5

Specificity results for Protocols A through E using analytical samples C-1 and C-2, healthy tomato seed sample D, and healthy pepper seed sample E[a]

	Protocol				
Sample ID	A (*n*)	B (*n*)	C (*n*)	D (*n*)	E (*n*)
Sample C-1	83.3 ± 13.9 (18)	100.0 ± 1.6 (27)	92.6 ± 4.1 (27)	100.0 ± 3.9 (18)	100.0 ± 3.9 (18)
Sample C-2[b]	77.8 ± 10.3 (18)	100.0 ± 1.6 (26)	91.7 ± 3.8 (27)	94.4 ± 11.1 (18)	100.0 ± 5.1 (18)
Sample D	100.0 ± 4.6 (27)	100.0 ± 1.8 (25)	100.0 ± 2.8 (26)	95.8 ± 11.9 (24)	100.0 ± 4.6 (27)
Sample E	96.3 ± 11.3 (27)	77.8 ± 3.6 (26)	100.0 ± 3.5 (27)	100.0 ± 4.4 (24)	100.0 ± 4.6 (27)

[a] Numbers are percent negative and range of error, expressed as the 95% confidence interval (CI); for values at 100%, binomial distribution was used to estimate the error range.
[b] Sample C-2 is a 1/100 dilution of sample C-1.

laboratories that met the passing criteria described in the "Experimental design and panel composition" section and determined as the difference between the average cutoff value for the six laboratories (35.46) and the measurement of uncertainty (1.42) (Supplementary Table S4). The measurement of uncertainty was calculated as ks + U, where **k** is the expansion coefficient, **s** is the standard error of the six curves, and **U** is the mean value. The average of 34.04 C_q was rounded to 34 for simplicity.

Data from the three remaining laboratories could not be used for the cutoff calculations.

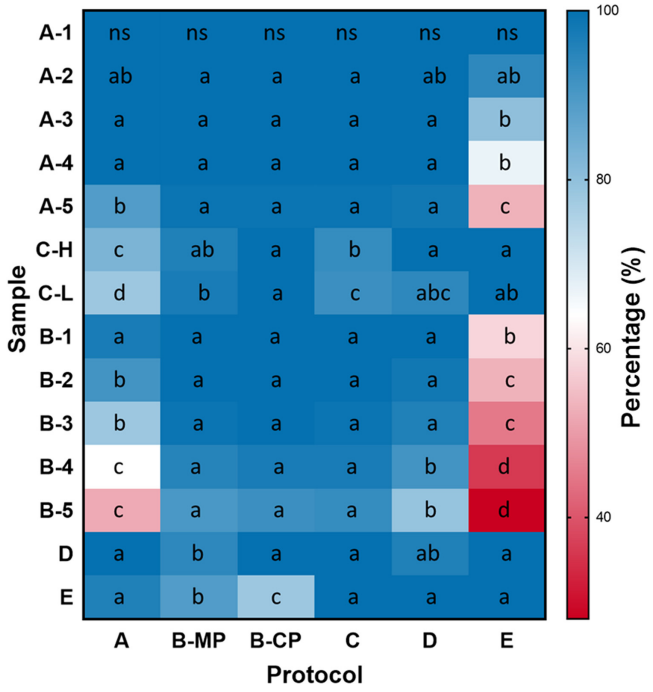


FIGURE 3

Limit of detection (LOD) values for all protocols with the in vitro tomato brown rugose fruit virus sample are shown on the left side of the graph. LOD values of all protocols with infested seed samples (sample B) at different dilutions are shown in the center graph, and the corresponding mean comparison results are expressed as a heat map shown on the right. Welch's analysis of variance test and a Dunnett's T3 test were used to assess mean differences across the different dilutions for each protocol. Letters group statistically similar data at $\alpha = 0.05$. The blue-to-red scale represents differences as percentages. MP, movement protein-encoding gene; CP, coat protein-encoding gene.

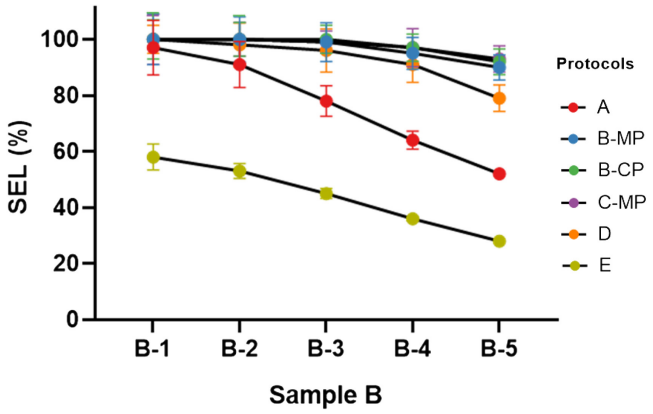


FIGURE 4

Sensitivity of samples B-1, B-2, B-3, B-4, and B-5 (5-point serial dilution) with each tested protocol. MP, movement protein-encoding gene; CP, coat protein-encoding gene.

Likelihood ratios

All tests were observed by global accuracy (total correct/total attempts), positive and negative predictive value (correct positives/total positives and correct negatives/total negatives), and likelihood ratio. Likelihood ratios are presented in this paper (Table 8).

TABLE 6

Coefficient of variation (CV) for parameters used to determine precision in real-time RT-PCR Protocols B and C[a]

Protocol	Repeatability, %CV (*n*)	Intermediate precision, %CV (*n*)	Reproducibility, %CV (*n*)
B MP	1.65 (97)	3.55 (25)	8.57 (13)
B CP	1.53 (102)	3.73 (25)	8.58 (14)
B nad5	1.43 (69)	4.38 (30)	5.11 (14)
C MP	1.85 (107)	4.01 (8)	8.68 (14)
C nad5	1.67 (54)	3.73 (6)	7.73 (8)

[a] The number of contributing data points (*n*) represents %CV results contributed by each sample dilution to observe combined variation. The precision for each sample or sample dilution is shown in Supplementary Table S2. The table shows the %CV around each mean specific to each sample (range of error). In this case, the %CV can be used to back-calculate to the standard deviation (%CV/100 = s/mean). Use the formula %CV/100 × mean. False reactions are not included in precision calculations. (*n*), the number of data points contributing to the percent error; MP, the tomato brown rugose fruit virus movement protein-encoding gene; CP, the coat protein-encoding gene; nad5, the mitochondrial nad5 plant gene.

TABLE 7

Linearity, accuracy, and assay cutoff values for the instruments used across six participating laboratories[a]

Laboratory	Linearity, r^2	Accuracy	Assay cutoff (*y*-intercept; C_{q0})
1	0.9957	–3.18	37.57
2	0.9983	–3.38	40.20
3	1.0000	–3.33	40.27
4	0.9964	–3.37	36.27
5	0.9967	–3.48	38.84
6	0.9986	–3.27	39.56

[a] Good precision is indicated with an r^2 value greater than 0.98. Accuracy (calibration curve slope) and assay cutoffs are expected at -3.32 ± 0.3 and 40 ± 4, respectively. All results obtained were within the expected ranges. Amplification efficiency can be calculated using the formula 1/10^(1/slope)-1, expressed as a percentage (e.g., for laboratory 1, −3.18 = 106%).

TABLE 8

Likelihood ratios for Protocols A to E[a]

Protocol (target region)	Positive likelihood ratio (PLR)	Negative likelihood ratio (NLR)
A (MP)	28	0.49
B (MP and CP)	17	0.08
C (MP)	29	0.07
D (CP)	38	0.22
E (RdRP)	6	0.72

[a] Protocol B was designed to detect for the movement protein (MP) and coat protein (CP) encoding RNA. Values were estimated for samples B, D, and E. RdRP, RNA-dependent RNA polymerase-encoding gene.

Positive (PLR) and negative (NLR) likelihood ratios are used to reflect the effectiveness of each test. The closer these values are to 1, the more difficult it is for the test to distinguish positive and negative samples. A PLR above 5 and an NLR below 0.5 confirmed the capacity for distinguishing positive from negative samples. The PLR and NLR are calculated using the formulas sensitivity/(1 − specificity) and (1 − sensitivity)/specificity. Protocols A through D had PLR ratios ranging from 17 to 38 and NLR ratios ranging from 0.07 to 0.49. Protocol E had 6 (PLR) and 0.72 (NLR) likelihood ratios.

Discussion

Five PCR-based protocols were assessed by nine laboratories and compared for their ability to detect ToBRFV in tomato and pepper seeds. The project goal was to determine which protocols' performance characteristics were optimal and generated similar results on the same sample set so that they could be used in the NAPPO region as regulatory diagnostic tools for ToBRFV to prevent its introduction and spread in the NAPPO region.

The Euphresco test performance studies for the detection of ToBRFV in seed (Giesbers et al. 2021) compared serological (ELISA) tests and a variety of molecular methods, including real-time RT-PCR, end-point RT-PCR, and isothermal amplification tests such as loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA). Only end-point and real-time RT-PCR protocols were selected for the NAPPO ring test because they are faster and user-friendly, and they have the best sensitivity and specificity, as shown by Giesbers et al. (2021). From around 20 protocols reviewed, five were selected for the ring test. Each of these five protocols (Table 1) was validated to different extents prior to the ring test. Protocol B and primers of Protocol E were also included in the Euphresco test performance studies (Giesbers et al. 2021; Luigi et al. 2022). The cycle cutoff used to determine positive versus negative results in the real-time RT-PCR was suggested for Protocols B and C during their respective validation studies. A cutoff of 32 has been suggested for Protocol B (ISF ISHI-Veg 2024). The protocol C cutoff was estimated at 34 based on extensive validation studies of protocols C and D conducted by the PPCDL (USDA 2019a). Protocols can be requested by emailing APHIS-PPQCPHSTBeltsvilleSampleDiagnostics@usda.gov.

The ring test scheme evaluated the performance of these protocols, using parameters including LOD, sensitivity, specificity, and precision, in different laboratory facilities with varied instrumentation. The ring test panel consisted of analytical (samples A and C) and diagnostic (samples B, D, and E) samples. All laboratories received blind samples and known controls from the same homogenous materials. Laboratories were also provided with the same RT-PCR reagents. The array of sample matrices evaluated in different laboratory environments allowed for granular analysis of test robustness. Controls were selected based on the ring test schematic to gather information on post-extraction sample handling of healthy and ToBRFV-infested seeds and the accurate measurement of nucleic acids using different laboratory equipment and process flows in environs free of contamination.

Nine laboratories from NAPPO countries participated in the ring test, generating 3,680 data points, which were used to analyze performance characteristics, as discussed below. The definitions of sensitivity and LOD can differ depending on what international standard is followed or what diagnostic network is modeled. In this ring test, the two parameters were distinctly separated by calculation to observe a situation in which one might not be indicative of better performance over the other. As an example, Protocol D sample B-5 had a higher percent positive rate than Protocol B-MP. This might suggest that Protocol D is a more reliable protocol with an improved LOD. However, because Protocol B-MP had an improved percent positive rate at higher concentrations than Protocol D, Protocol B-MP exhibits better sensitivity than Protocol D for all five dilutions (B-1 to sample B-5) (Table 4). Therefore, both LOD and sensitivity were defined to evaluate protocol fitness, taking into consideration two different traits that, although not discrete, compare tests from different perspectives. In fact, as measured in the NAPPO ring test, sensitivity is likely more reliable than LOD; due to the varying nature of LOD measurements (e.g., sample type, operator, instrument calibration status, and reagent lots), more weight should be placed on percent positivity over the range of concentrations, representing a broader view of assay amplification kinetics.

Four protocols (A, B, C, and D) out of the five demonstrated comparable sensitivity. This analysis also confirmed that real-time RT-PCR Protocols B and C are superior to the end-point protocols. These findings echo the Euphresco study that showed real-time RT-PCR as the most sensitive to detect ToBRFV (Giesbers et al. 2021).

Results of the analytical sample C (ToMMV) and healthy tomato (D) and pepper seed (E) samples were used to evaluate the specificity of each protocol. Protocol B sample E showed a statistical difference when compared with the other protocols for the MP target. There was no statistical difference between samples C-1 and C-2 for each protocol, indicating no cross-reactivity with ToMMV within each protocol's amplification kinetics. If cross-reactivity were present, sample C-1 would have a statistically significant lower rate of true negatives than sample C-2. There was a noticeable difference in percent negatives when comparing samples C-1 and C-2 with samples D and E for Protocol A, but the difference was not statistically significant.

Implementation of the calibrator curve had the added benefit of finding the real-time PCR amplification efficiency, linearity, and cycle cutoff for each laboratory test run. Cycle cutoff sets the Cq range for positive and negative interpretation within a laboratory's specific internal system (equipment and reagents). Adhering to this line of thought, Euphresco and EPPO (Giesbers et al. 2021) did not propose a uniform cutoff but left it to each lab to determine. Laboratory results can be qualitatively compared using binary analysis, but uniform cutoffs are challenging to establish unless the same homogenous material is used for all laboratories and a calibrator curve standardizes each test run. The NAPPO ring test utilized both common material batches distributed from a single location and a well-characterized calibrator curve (Supplementary Table S4). Although three laboratories did not produce data that could be applied toward a cutoff estimation, six laboratories did, resulting in a predicted cutoff of 34 for both real-time protocols. When this cutoff was applied to the data of the three remaining laboratories, it reliably distinguished positive from negative samples for two of them. Thus, positive and negative samples for 8 out of 9 laboratories were reliably identified using a cutoff of 34 in this study. A review of the results of Giesbers et al. (2021) revealed that Cq values up to 34 were considered reliably positive, although a cutoff was not applied. Bernabé-Orts et al. (2021) suggested that a cutoff of 35 could be appropriate for the ISHI-Veg and their Abiopep protocols when used with plant RNA. Although the cutoff estimated in our study aligns well with these results, it is advisable for each laboratory to conduct additional validation studies in their respective testing environment using well-characterized reference material to establish a cutoff.

A plant gene was used as an internal control in the real-time PCR protocols, adding the benefit of evaluating the quantity and quality of the total RNA extracted, including any viral target

RNA, in the same test tube. Protocol B targets two virus regions (MP and CP), thus providing better confidence in the results than Protocol C, with one viral target (MP). However, Protocol B target CP exhibited cross-reactivity with healthy pepper seeds.

Of the three end-point RT-PCRs, Protocol D demonstrated the best performance compared with the other two (A and E), with LOD and sensitivity closer to the real-time protocols than the end-point protocols. This assay targets the CP gene, which is located not only in the genomic RNA but also in two subgenomic RNAs (Salem et al. 2023). The CP subgenomic RNA is transcribed at a higher rate due to its efficient promotor and its 3′ proximal location in the genome (Dawson and Lehto 1990). The abundance of target copies may explain its superior sensitivity. On the contrary, Protocol E targets the RNA-dependent RNA polymerase-encoding gene, located on genomic RNA only, thus limiting copy numbers of the target, which results in a less desirable LOD. Protocol E showed exceptional specificity when tested with closely related species and ToBRFV-free samples. Nevertheless, better specificity does not offset both the low sensitivity and higher testing complexity of Protocol E. Protocol E requires two-step RT-PCR amplifications in two separate reactions for a viral and plant target, limiting the throughput of the detection.

Based on the NAPPO ring test analyses, the Expert Group recommended three protocols to the NAPPO Executive Committee: Protocol B, developed by ISF ISHI-Veg/NSHS, and two USDA-APHIS protocols, Protocol C (primers from Chanda et al. 2021) and Protocol D (primers from Dey et al. 2021). The three recommended protocols were found comparable and best fit for the detection of ToBRFV in tomato and pepper seeds. The end-point Protocol D lacks an internal control that may limit its applicability for the detection of ToBRFV. However, this protocol might be used as a follow-up of the real-time protocol to confirm the presence of the virus with a different genomic target or serve as a cost-effective option for laboratories without real-time PCR equipment. It also allows for sequencing of the PCR product if further confirmation of the results is needed.

No efforts were made to harmonize the RNA extraction protocols in this study. However, results from Protocols B, C, and D were comparable across nine participating laboratories using different RNA extraction protocols, including manual or robotic extraction, and different thermocyclers (Applied Biosystems Incorporated, Bio-Rad, or Rotorgene for real-time; Qiagen, Biometra, or Eppendorf for end-point), suggesting fitness for use across the NAPPO region. Protocol B performed the best in both EPPO and NAPPO studies (Giesbers et al. 2021 and this work), providing grounds for ToBRFV seed testing harmonization between country members of these organizations.

Harmonizing diagnostic protocols would facilitate the safe trade of tomato and pepper seeds in our region and avoid unnecessary retesting by the exporting and importing countries. The ring test experimental design used in this study can serve as a model for future studies to evaluate new protocols being developed for detecting ToBRFV or other seed-transmitted pathogens. Consideration should be given to inviting countries outside the NAPPO region to participate in future ring test studies, particularly those with significant seed trade with the NAPPO member countries. The control of any high-consequence pathogen requires the implementation of an integrated approach, including cultural practices, new germplasm, and cooperation within the region; however, a reliable diagnostic test is the first step toward the safeguarding continuum. Harmonizing diagnostic protocols that produce comparable results, although challenging, facilitates trade, as trading partners may recognize each other's testing results. The costs and time associated with retesting and trade delays are decreased, resulting in cheaper, faster, and more predictable trade.

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