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Primer Modification Improves Rapid and Sensitive *In Vitro* and Field-Deployable Assays for Detection of High Plains Virus Variants

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A high consequence pathogen, *High plains virus* (HPV) causes considerable damage to wheat if the crop is infected during early stages of development. Methods for the early, accurate, and sensitive detection of HPV in plant tissues are needed for the management of disease outbreaks and reservoir hosts. In this study, the effectiveness of five methods—real-time SYBR green and TaqMan reverse transcription-quantitative PCR (RT-qPCR), endpoint RT-PCR, RT-helicase dependent amplification (RT-HDA) and the Razor Ex BioDetection System (Razor Ex)—for the broad-range detection of HPV variants was evaluated. Specific PCR primer sets and probes were designed to target the HPV nucleoprotein gene. Primer set HPV6F and HPV4R, which amplifies a product of 96 bp, was validated *in silico* against published sequences and *in vitro* against an inclusivity panel of infected plant samples and an exclusivity panel of near-neighbor viruses. The primers were modified by adding a customized 22 nucleotide long tail at the 5′ terminus, raising the primers' melting temperature (T_m; ca. 10°C) to make them compatible with RT-HDA (required optimal T_m = 68°C), in which the use of primers lacking such tails gave no amplification. All of the methods allowed the detection of as little as 1 fg of either plasmid DNA carrying the target gene sequence or of infected plant samples. The described *in vitro* and in-field assays are accurate, rapid, sensitive, and useful for pathogen detection and disease diagnosis, microbial quantification, and certification and breeding programs, as well as for biosecurity and microbial forensics applications.

The disease caused by *High plains virus* (HPV) was first described in 1993 in corn and wheat crops in the High Plains region (Texas, Kansas, Idaho, Colorado, Nebraska, and Utah) of the United States (1, 2). The virus has since been found in Florida (USA), Israel, Brazil, Chile, and possibly China and Australia (3). High plains disease (HPD) symptoms may be confused with those caused by *Wheat streak mosaic virus* (WSMV; genus *Tritimovirus*), which has a similar but wider host range (4). HPV is transmitted by wheat curl mites (*Aceria tosichella*) (5). The host range of HPV includes cheat grass (*Bromus secalinus* L.), wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), oat (*Avena sativa* L.), rye (*Secale cereale* L.), maize (*Zea mays* L.), and some weeds, including yellow foxtail (*Setaria glauca* L.) and green foxtail (*Setaria viridis* L.) (4). HPV causes chlorotic spots and mosaic and general yellowing on wheat and can be followed by stunting. Chlorosis with flecking or streaking and reddening of leaf margins has been reported on maize. In severe cases, mortality has been observed (1–3).

Lebas et al. (6) developed a procedure for inspecting sweet corn seed shipments by testing germinated seedlings in quarantine using reverse transcriptase PCR (RT-PCR). However, the number of recognized HPV variants has risen in recent years (7, 8). HPV has a number of variants and can form complexes with other viruses (7). Wheat plants showing systemic mosaic symptoms (March and April) may not react to antibodies developed for WSMV or HPV (8). HPV disease was associated with early volunteer, and mites moved onto it readily from the old crop. The most severe disease outbreaks are found where volunteer wheat provides a "green bridge" through the summer between successive wheat crops (9). These circumstances result in a need for a broad-range detection method suitable for diverse HPV variants in field settings to assist integrated pest management decision-making.

PCR-based techniques, which are generally more sensitive than immunological methods and have high specificity and powerful discriminatory capabilities, are most widely used (10). Quantitative PCR (qPCR)-based methods eliminate the need for post-PCR product analysis (electrophoresis) and, among these formats, TaqMan and SYBR green qPCR are the most widely used (11). The TaqMan system has a slight advantage because the SYBR green dye binds to any double-stranded DNA, possibly resulting in nonspecific fluorescence (12). The implementation of these techniques in Extension and other applied programs has been increasing yearly (13). Endpoint RT-PCR (6), multiplex RT-PCR (14), and RT-qPCR (15) also have been developed for the detection of a variety of plant viruses.

In a field setting, detecting viruses with high sensitivity and specificity can be challenging. Currently, there is no on-site, rapid, reliable, and sensitive detection method for HPV. Antibodies are commercially available for HPV detection by double antibody sandwich enzyme-linked immunosorbent assay. However, this method is inconvenient to carry out in the field because of the need for microtiter plates, refrigerated reagents, a microtiter plate reader, and access to electricity. Use of the Razor Ex BioDetection System (Razor Ex; Idaho Technology, Inc., Salt Lake City, UT), a portable, battery operated, compact real-time qPCR system, allows sensitive in-field pathogen detection by a minimally trained operator without the need for a laboratory facility. Other portable thermocyclers include the Smart Cycler (Cepheid, Sunnyvale,

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CA), the LightCycler (Roche Applied Science, Indianapolis, IN), and the Bio-Seeq instrument (Smiths Detection, Edgewood, MD), but the Razor Ex has the advantages of very compact size (11 lb) and rapid cycling (only 30 to 40 min) (10, 16). Originally, the Razor Ex was designed for military use, to detect high-consequence organisms on-site. The use of ready-to-use, freeze-dried reagent pouches and barcode-based PCR cycling programs minimize the risk of cross-contamination during assay performance.

The use of isothermal virus detection methods is gaining wide popularity because they provide an alternative method for nucleic acid amplification with no need for sophisticated and expensive thermocyclers and can be performed using a heat block or water bath at just two constant temperatures (17, 18). Loop-mediated isothermal amplification has been reported for plant virus detection (19–22), However, there are only a few reports of the use of RT-helicase dependent amplification (RT-HDA) detection with plant-pathogenic viruses (23) and other viruses (24, 25), and these applications required the use of a primer with a T_m of 68°C. Hence, there is a need for new primer sets for RT-HDA or for modification of the existing primer set to raise the T_m. Arif and Ochoa-Corona (26) reported that the addition of A/T-rich sequences at the 5′ primer terminus can both broaden the T_m range for PCR amplification and increase the assay sensitivity.

The aims of this research were to develop and compare the efficacy, *in vitro*, of five different DNA amplification methods: SYBR green RT-qPCR, TaqMan RT-qPCR, endpoint RT-PCR, the field deployable Razor Ex system, and RT-HDA, using a single primer set containing 5′ modification for broad-range detection of different HPV variants. The results will facilitate investigator selection of the assay most appropriate for their needs and available facilities, and enhance investigative capability for agricultural biosecurity and microbial forensics. Primer modification to meet the required T_m of a particular technique could be useful for other assays as well and could eliminate the need to design and validate multiple primer sets for different assays.

MATERIALS AND METHODS

Exclusivity panel. Nine wheat samples positive for WSMV and/or *Triticum mosaic virus* (TriMV) but negative for HPV were used in the exclusivity panel. The samples were collected from Oklahoma (sample numbers 201100880, 200900425, 200900409, 200900071, and 200800354), Montana (sample numbers MT 08-3, MT 08-10, and MT 08-57), and Colorado (sample number CO 08-11) and stored at −80°C. These samples were prescreened using a multiplex PCR developed and validated in our laboratory to detect and differentiate HPV, WSMV, and TriMV (unpublished data). Positive controls for other different viruses, including *Impatiens necrosis spot virus* (INSV), *Tomato spotted wilt virus* (TSWV), *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus* (TMV), *Hosta virus X*. (HVX), (Agdia, Elkhart, IN), and *Triticum aestivum* (a host plant) were also used in the exclusivity panel for specificity assays.

Inclusivity panel and RNA isolation. Symptomatic and asymptomatic infected wheat leaves used in inclusivity panel for specificity assays were collected from Oklahoma, Kansas, Texas, Montana, North Dakota, and Colorado and stored at −80°C. A list of HPV-infected wheat plant samples is given in Table 1. Total RNA was isolated from symptomatic and asymptomatic wheat leaves, as well as INSV, TSWV, CMV, TMV, and HVX, using an RNeasy plant minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Primer and probe design. Specific PCR primers and probe were designed targeting the HPV nucleoprotein gene, whose gene sequences were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/). The primers HPV6F (5′-GCAACAAGAGGTATGAGGACAA-3′) and HPV4R (5′-TCCTTAGAAAAACCAGCACTCA-3′) and the probe HPV-P2 (5′-TTCAATGAGGATGACGACTTCATGAACAA-3′) were designed from National Center for Biotechnology Information (NCBI) gene accession U60141 (Fig. 1) using the Web-interface applications Primer3, mFold, and BLASTn and validated thermodynamic parameters (26). Internal secondary structures and self-dimers were determined using mFold (27). Primers were aligned with HPV nucleoprotein gene accession numbers KC337342, KC337341, AY836525, AY836524, and U60141 to confirm the broad-range detection capabilities of the designed primers and probe. The specificity was confirmed *in silico* by aligning the primer and probe sequences using BLASTn, available at the NCBI GenBank database. Primers HPV3F, HPV4F, HPV5F, and HPV3R were also designed targeting a similar gene sequence, following the same protocol mentioned above, to compare their broad-range detection capabilities (Fig. 2). Probes labeled with reporter and double-quencher dyes 5′ 6-carboxyfluorescein/ZEN/3′ Iowa Black FQ (5′6-FAM/ZEN/3′IBFQ) and primers were synthesized by IDT (Integrated DNA Technologies, Inc., Coralville, IA).

Primer modification for RT-HDA. Primers HPV6F and HPV4R failed to meet the primer T_m of 68°C, recommended by the manufacturer for the IsoAmp III Universal tHDA kit (BioHelix, Beverly, MA). Therefore, both primers were modified by adding customized 22-nucleotide tails (5′-ATACTACTATACTATATCATAT-3′) at the 5′ terminus (Fig. 1) to raise the basic T_m by about 10°C, a level compatible for RT-HDA. The primer's T_m was assessed using online software (http://mbcf.dfci.harvard.edu/docs/oligocalc.html).

cDNA synthesis. First-strand cDNA synthesis was performed, using random hexamer primers, on RNA extracted from symptomatic and asymptomatic wheat leaves, as well as from all positive controls used in the exclusivity panel to assess the range of detection and cross-reactivity of the primers. Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) were used according to the manufacturer's instructions. From each sample, 4 μl of RNA template was used to synthesize the first-strand cDNA.

Endpoint RT-PCR. Endpoint RT-PCR assays were carried out in 20-μl reaction mixtures containing 10 μl of GoTaq Green Master Mix (Promega), 1 μl (5 μM) each of primers HPV6F and HPV4R, 2 μl of cDNA as a template, and 6 μl of nuclease-free water (Ambion, Austin, TX). Reactions were performed in an Eppendorf thermal cycler (Eppendorf, Hauppauge, NY). The cycling parameters consisted of an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 52°C for 30 s, and extension at 72°C for 30 s. A final extension was performed for 3 min at 72°C. Positive (known HPV-infected and/or plasmid DNA carrying the target gene sequence) and negative (nontemplate control; water) controls were included in each round of PCR amplification. All 20 μl of the reaction volume was electrophoresed in a 1.5% agarose gel in 1× TAE buffer. The product size was determined using a 1-kb plus ladder (Invitrogen).

Real-time SYBR green RT-qPCR. RT-qPCR amplification was carried out in 20-μl reaction mixtures containing 10 μl of Platinum SYBR green qPCR SuperMix-UDG (Invitrogen), 0.8 μl (5 μM) each of primers HPV6F and HPV4R, 2 μl of template cDNA, and 6.4 μl of nuclease-free water. Positive and negative controls were included in each round of PCR amplification, and each reaction was performed in three replicates. The cycling parameters included two initial holds each for 2 min at 50 and 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s.

Real-time TaqMan RT-qPCR. RT-qPCR amplifications with the primer set HPV6F and HPV4R was carried out in 20-μl reaction mixtures containing 10 μl of Platinum quantitative PCR SuperMix-UDG (Invitrogen), 0.8 μl (5 μM) of each forward and reverse primer, 0.2 μl (5 μM) of HPV-P2 probe, 2 μl of template cDNA, and 6.2 μl of nuclease-free water. Positive and negative controls were included in each round of qPCR amplification, and each reaction was performed in three replicates. Cycling parameters included two 2-min initial holds at 50°C and 95°C, followed

TABLE 1 Description of HPV-infected wheat plant samples used in protocol validation collected from different states in the United States

Sample code	Collection location	Detection technique[a]				
		Endpoint RT-PCR	TaqMan RT-qPCR	SYBR green RT-qPCR	RT-HDA	Razor Ex
UA04-143	Kansas	+	+	+	+	+
HPV 404-82	Kansas	+	×	+	+	+
HPV 404-83	Kansas	+	+	+	×	×
HPV 407-961	Kansas	+	+	+	+	+
HPV 08-01	Kansas	+	+	+	+	+
121	Kansas	+	+	×	×	×
Kans 08-117	Kansas	+	+	×	×	×
406A	Kansas	+	+	+	+	+
Wheat Infected-26	Kansas	+	+	×	+	+
Wheat Infected-27	Kansas	+	×	×	+	+
201101040	Oklahoma	+	×	×	×	×
201101042	Oklahoma	+	×	×	×	×
201000496	Oklahoma	+	×	×	×	×
200800256	Oklahoma	+	×	×	+	×
406A1	Oklahoma	+	+	+	+	+
406A2	Oklahoma	+	+	+	+	+
Tx09-144	Texas	+	+	×	+	×
Tx09-57	Texas	+	+	×	×	×
Tx09-58	Texas	+	+	×	×	×
Tx08-13	Texas	+	+	×	×	×
Tx08-25	Texas	+	+	×	×	×
Tx09-81	Texas	+	×	×	×	×
Tx09-66	Texas	+	×	×	×	×
Tx08-4	Texas	+	×	×	×	×
PP0900966	North Dakota	+	+	×	×	×
PP0900896	North Dakota	+	×	×	×	×
MT 08-12	Montana	+	×	×	×	×
MT 08-56	Montana	+	×	×	×	×
MT 08-317	Montana	+	+	×	+	+
CO 08-9	Colorado	+	×	×	×	×
APC control	KC555272[b]	+	×	+	+	×
Positive control	Generated in NIMFFAB[c]	+	+	+	+	+
Nontemplate control (nuclease-free water)	Ambion, Austin, TX	–	–	–	–	–

[a] ×, sample not tested; +, positive for HPV; –, negative for HPV.
[b] The multitarget artificial positive control (APC), obtained from the NIMFFAB laboratory, contains all of the target diagnostic (for specific primers) sequences of the pathogen; the sequence of APC was submitted to GenBank under accession number KC555272. SYBR green reactions with infected samples were performed in a single replicate.
[c] Positive controls containing the target gene sequence were generated in the NIMFFAB laboratory, Stillwater, OK.

by 40 cycles at 95°C for 15 s, and 60°C for 45 s. The RT-qPCR assays were performed in a Rotor-Gene 6000 thermocycler. Rotor-Gene 6000 series software 1.7 (Built 87) having a manual cycle threshold (C_T) of 0.1 was used for data analysis (Corbett Research, Sydney, Australia).

Razor Ex BioDetection System. Razor Ex amplification was performed in 150-μl reaction mixtures containing 75 μl of Platinum quantitative PCR SuperMix-UDG, 6.0 μl (5 μM) each of primers HPV6F and HPV4R, 1.5 μl (5 μM) of HPV-P2 probe, 4 μl of template cDNA, and 57.5 μl of nuclease-free water. Because Razor Ex freeze-dried reagent pouches are not commercially available for HPV, we inserted standard TaqMan reagents into empty pouches. Positive and negative controls were included in each round of Razor Ex amplification. The rapid cycling parameters consisted of an initial hold for 2 min at 50°C and then a first cycle at 94°C for 4 min and 60°C for 15 s, followed by 54 cycles of 91°C for 3 s and 60°C for 15 s. The barcode used to upload the cycling parameters (Fig. 3) can be scanned by readers directly from this paper. The Razor Ex BioDetection System was used to perform the assays.

RT-helicase-dependent amplification. The modified primer set HPV6F.LF/HPV4R.LF was used for RT-HDA, and reactions were carried out in 25-μl volumes using an IsoAmp III universal tHDA kit. Two separate reaction mixes, A and B, were prepared. Mix A contained 1.25 μl of 10× annealing buffer II, 0.5 μl (5 μM) of each forward (HPV6F.LF) and reverse (HPV4R.LF) primer, 3 μl of template cDNA and 7.25 μl of nuclease-free water. Mix B contained 1.25 μl of 10× annealing buffer II, 1 μl of $MgSO_2$ (100 mM), 2 μl of NaCl (500 mM), 1.75 μl of IsoAmp deoxynucleoside triphosphate solution, 1 μl of IsoAmp enzyme mix, and 5.5 μl of nuclease-free water. Each was gently mixed by pipetting. Mix A was incubated for 2 min at 95°C and immediately transferred onto ice. Mix B was added to mix A, and the new mixture was incubated at 63°C for 100 min. An optional last step of 95°C for 3 min was added to denature the enzyme. The positive and negative controls were included in each round of amplification. After amplification, electrophoresis was performed as mentioned above.

Sensitivity assays. To determine the assay detection limits using primer set HPV6F/HPV4R or HPV6F.LF/HPV4R.LF and probe HPV-P2, two sensitivity assays were performed with each method except the Razor Ex. A known quantity of plasmid DNA was used to quantify the target pathogen genomic nucleic acid with RT-qPCR, using cDNA synthesized from infected plant RNA. A standard graph was generated using target HPV cDNA and/or plasmid DNA, serially diluted in 10-fold increments,

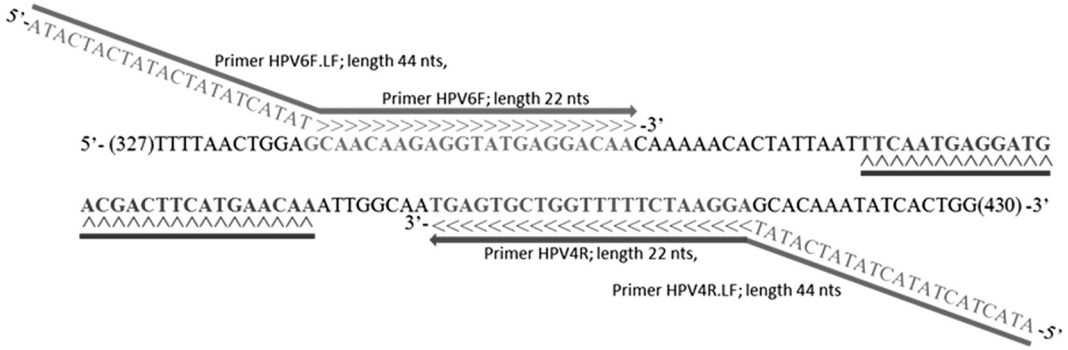

FIG 1 Addition of an AT-rich tail at the 5′ terminus of each primer. Primers HPV6F and HPV4R were modified with a customized 22 nucleotide tail at the 5′ terminus, which increased the T_m value of each primer by about 10°C and making the primer set compatible with RT-helicase dependent amplification in isothermal conditions. The addition of this tail to both forward and reverse primers also increased the final amplified product size (96 + 22 + 22 = 140 bp). >, forward primer (HPV6F); <, reverse primer (HPV4R); ^, probe (HPV-P2).

and used at 1 ng to 1 fg per reaction. Each RT-qPCRs was performed in three replicates.

Positive control. A positive control was generated by cloning a partial gene sequence (298 bp) of HPV nucleoprotein, amplified using primer set HPV4F and HPV3R, which amplifies the target gene sequence for all of the designed HPV primers and probes, including HPV6F, HPV4R, and HPV-P2 (Fig. 2). Amplicons were eluted from the agarose gel using Quantum Prep Freeze 'N Squeeze spin columns (Bio-Rad, Hercules, CA) and cloned into a plasmid vector (pCR2.1-TOPO) using a TOPO-TA cloning kit (Invitrogen). Plasmid DNA carrying target gene sequence were purified from overnight grown bacterial cultures using a QIAprep spin miniprep kit (Qiagen) and was sequenced by the Oklahoma State University Recombinant DNA/Protein Resource Facility using M13F primer. Our laboratory's multitarget artificial positive control (GenBank accession number KC555272; unpublished data) also contains all of the above primer sequences, which were artificially synthesized and inserted into pUC57 (GenScript USA, Inc., Piscataway, NJ). The concentrations of total RNA and plasmid DNA were measured using a NanoDrop v.2000 spectrophotometer (Thermo Fisher Scientific, Inc., Worcester, MA).

Nucleotide sequence accession number. The nucleoprotein gene sequence amplified using primer set HPV4F and HPV3R was deposited in GenBank under accession number KF031532.

RESULTS

***In silico* specificity primers and probe.** All designed primers showed a broad range of detection of HPV variants. However, primer combination HPV6F/HPV4R was selected for the present study because of its smaller product size (96 bp; Fig. 2), which is ideal for the qPCR, Razor Ex, and RT-HDA methods. Primer set HPV6F (T_m = 59°C; 45% G+C content; 22 bp) and HPV4R (T_m = 59°C; 41% G+C content; 22 bp) and probe HPV-P2 (T_m = 69°C; 38% G+C content; 29 bp), evaluated *in silico*, were found to be specific for HPV, with calculated E values of 0.008 for both the forward and the reverse primer and 1e−06 for the probe. Both primers HPV6F and HPV4R and probe HPV-P2 met the desired 100% query coverage and showed 100% identity after an alignment using BLASTn with HPV nucleoprotein gene accession numbers KC337341, KC337342, AY836524, AY836525, and U60141 in the NCBI GenBank nucleotide database. No matches with other plant virus or bacteria were detected. The ΔG plot values, calculated using mFold, were 0.9, 0.8, and 0.0 kcal/mol, and the numbers of predicted secondary structures were 2, 6, and 1, for primer HPV6F and HPV4R and probe HPV-P2, respectively. None of the secondary structure formation occurred at the 3′ terminus. The double-quenched probe contains IBFQ quencher at the 3′ terminus and an internal ZEN quencher, which helps to maximize the signal and minimize the background.

Addition of 5′ tail. The 59°C T_m of primers HPV6F and HPV4R was increased about 10°C by adding a customized, 22-nucleotide tail at the 5′ terminus (Fig. 1). This modification increases assay sensitivity and enhances the RT-HDA compatibility

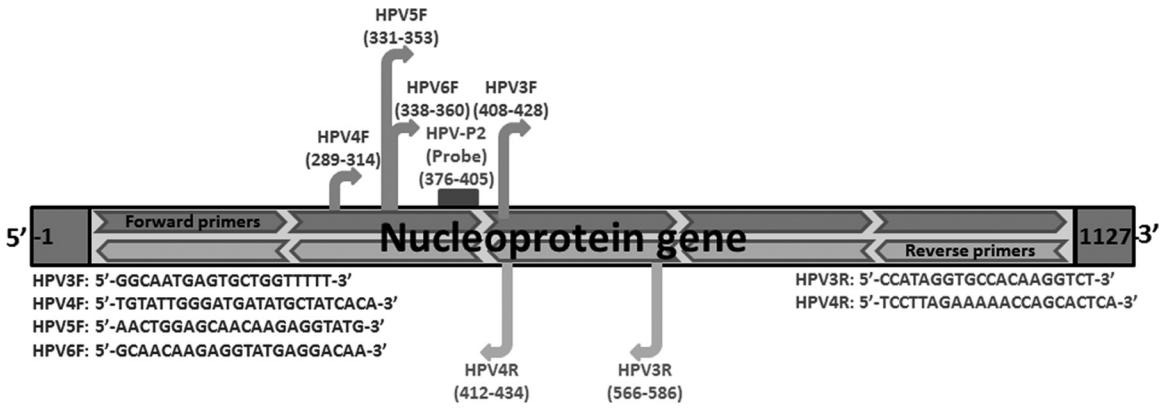

FIG 2 Location of HPV primers on nucleoprotein gene (GenBank accession number U60141). The different primers were designed to make the combination between different forward and reverse oligonucleotides to select the primer combination having the broadest-range detection capability.

FIG 3 Barcode for PCR cycling conditions. The rapid PCR cycling conditions for the Razor Ex BioDetection System can be uploaded using the bar code.

of the primer set. Adding the tail at the 5′ end of both forward and reverse primers also increased the size of amplified product (96 + 22 + 22 = 140 bp). The modification made it possible to use a single primer set for all five of the assays.

Assay specificity. Primer specificity assessment showed no cross-reactivity with WSMV- or TriMV-infected wheat samples or with viruses INSV, TSWV, CMV, TMV, and HVX. Inclusivity was tested against a number of HPV-infected plant samples collected from six Great Plains states, including Oklahoma, Kansas, Texas, Montana, North Dakota, and Colorado (Table 1). These samples were prescreened (except for samples 201000496, Tx09-58, and Tx08-25) for the presence of HPV using a multiplex PCR (unpublished data). Totals of 30, 17, 8, 12, and 10 HPV-infected samples were tested and found to be positive for HPV using endpoint RT-PCR, TaqMan RT-qPCR, SYBR green RT-qPCR, RT-HDA, and Razor Ex (Table 1). No contradictory results were obtained with any of the methods (Table 1).

Comparison and cost analysis. The costs of PCR reagents (master mixes, probe, and commercial kits for isolation) for the five assays were compared. Isolation of one RNA sample from infected plant tissue, using the RNeasy plant minikit, cost about $6.50, and synthesis of 10 μl of cDNA from the RNA was about $4, for a total of about $10.5 per sample. Similar costs were incurred for all of the techniques used in this research. In the second step of RT, the cost of amplification varied (Table 2), the Razor Ex being the most expensive at $144 per 12 reactions ($12/reaction), including the cost of Platinum quantitative PCR SuperMix-UDG, the TaqMan probe, and 12 empty pouches ($50 each). A minimum of $0.35 per reaction was calculated for endpoint RT-PCR, not counting the cost of electrophoresis. About $1.1 and $0.93 per reaction were calculated for TaqMan and SYBR green RT-qPCR, respectively (Table 2). The times required for completion of each assay after cDNA synthesis were approximately 25, 90, 90, 150, and 200 min for Razor Ex, TaqMan RT-qPCR, SYBR green RT-qPCR, endpoint RT-PCR, and RT-HDA, respectively. The Razor Ex assay is the most user-friendly, followed by endpoint RT-PCR, because these techniques require less operator training than TaqMan RT-qPCR, SYBR green RT-qPCR, and RT-HDA. TaqMan RT-qPCR, SYBR green RT-qPCR, and endpoint PCR required access to a laboratory facility, whereas RT-HDA and Razor Ex are field deployable. All of the techniques detected as little as 1 fg of target cDNA and plasmid DNA carrying the target gene sequence (Fig. 4 and 5, Table 2; Razor Ex was not tested for sensitivity). The sensitivity of SYBR green RT-qPCR was always ~2 C_T values higher than that using TaqMan RT-qPCR (Table 3 and Fig. 5).

DISCUSSION

We report the development and comparison of *in vitro* and field-deployable reliable, accurate, and sensitive endpoint RT-PCR, TaqMan RT-qPCR, SYBR green RT-qPCR, Razor Ex, and RT-HDA methods for the detection of HPV using a single primer set employing modification at 5′ terminus, targeting a small (96-bp) segment of the HPV nucleoprotein gene.

HPV, an economically important wheat pathogen in the Great Plains of the United States, causes considerable damage every year. Due to high genomic variability among HPV variants, previously existing RT-PCR methods (6) were not able to detect all of them. A new approach, using primer sets of different forward and reverse oligonucleotides, provided the capability for broad-range detection of all HPV variants (Fig. 2) tested, including UA04-143, HPV 404-82, HPV 404-83, HPV 407-961, HPV 08-01, and others from different states of the Great Plains (Table 1). We report the development of a single, thermodynamically competent primer pair that fits all PCR variants and provides additional flexibility to investigators, who can adapt the assay to different circumstances, including field deployment.

In RT-HDA, the T_m of the primer set should be close to 68°C to provide optimal conditions for helicase and polymerase activity. The addition of a 5′ tail to our initially ineffective primers made them compatible with RT-HDA. Arif and Ochoa-Corona (26) reported that the addition of a 5′ tail improved primer thermodynamics and ultimately enhanced detection sensitivity. The 5′ tail, added to suboptimal primers, can enhance assay sensitivity up to 100 times and also broaden the annealing temperature range (26). For example, working with genomic DNA of the insect *Lepinotus reticulates*, Arif et al. (18) detected only down to 1 ng using HDA but as little as 100 fg using SYBR green qPCR. In that study, the fact that the authors did not use a customized 5′ AT-rich tail to reach the recommended T_m could explain the low HDA sensitivity. This approach could also be used to enhance the reliability and sensitivity of multiplex PCR assays by matching the T_ms of different primers for compatibility.

Assay specificity, accuracy, and reproducibility are critical for applications in quarantine situations, microbial forensics, biosecurity, and diagnostics (10). The specificity of designed primers was confirmed *in silico* after positive BLASTn matching with five accessions and positive-endpoint RT-PCR with 30 HPV-infected

TABLE 2 Comparative evaluation of five HPV detection methods—real-time SYBR green and TaqMan RT-qPCR, endpoint RT-PCR, RT-HDA, and Razor Ex—for cost, detection limit, and run time

Assay	Cost ($)[a]		Field deployable	Detection limit (fg)	Assay run time (min)
	Per sample[b]	Equipment[c]			
Razor Ex	12*	38,500	Yes	NT[d]	25
RT-HDA	2.68	700	Yes	1	200
Endpoint RT-PCR	0.35	9,500	No	1	150
TaqMan RT-qPCR	1.10	35,000	No	1	90
SYBR green RT-qPCR	0.93	35,000	No	1	90

[a] The values here do not include the cost for electrophoresis, labor charges, storage, and any other consumables and indirect costs. Isolation of one RNA sample from infected plant tissue, using the RNeasy plant minikit (Qiagen), costs about $6.50, and synthesis of 10 μl of cDNA from the RNA cost about $4, for a total of about $10.50 per sample. The costs incurred for all of the techniques used in this research were similar.
[b] Includes only the cost of reagents (including probe) used for amplification, and this may vary from manufacturer to manufacturer. *, this cost also included the pouch cost ($50/12 reactions).
[c] The cost of equipment, except for Razor Ex, may also vary from manufacturer to manufacturer.
[d] NT, not tested.

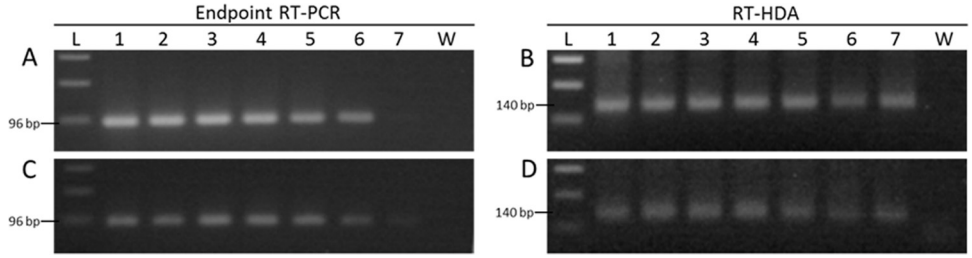

FIG 4 Sensitivity assays using endpoint RT-PCR and RT-helicase dependent amplification (RT-HDA). Target cDNA from HPV-infected wheat leaves and/or plasmid DNA carrying the target gene fragment was serially diluted in 10-fold increments and used at 1 ng to 1 fg per reaction. (A and C) Sensitivity assays using endpoint RT-PCR with infected cDNA (A) and plasmid DNA (C); (B and D) sensitivity assays using RT-HDA with infected cDNA (B) and plasmid DNA (D). Lane L, 1-kb ladder (Invitrogen). Lanes 1 to 7 contain serially diluted cDNA or plasmid DNA at concentrations from 1 ng to 1 fg. Lane W is NTC (nontemplate control; water).

plant samples collected from six states of the Great Plains (Table 1). Furthermore, we have demonstrated the specificity of five assays by the absence of false positives, false negatives, and cross-reactivity with other viruses. In this case, it was important that the assay detect a broader range of recently described HPV isolates than did previous tests, and our positive results with 30 HPV-positive samples from six U.S. states indicated that this criterion was achieved. No contradictory results were obtained when six randomly selected samples (UA04-143, HPV 407-961, HPV 08-01, 406A, 406A1, and 406A2) and a positive control (Table 1) were tested with all five techniques developed in this research.

Primer thermodynamics plays a significant role in assay sensitivity (26). These assays detect as little as 1 fg of target cDNA and the positive control (plasmid carrying the target gene sequence). Similar linear correlation (R^2), slope (Y), and reaction efficiency (Ex) values were obtained with independent TaqMan RT-qPCR and SYBR green RT-qPCR assays when generated using plasmid DNA (Table 3). Both the TaqMan RT-qPCR and the SYBR green RT-qPCR methods also showed exactly the same values when performed using cDNA (Table 3), demonstrating high accuracy, reliability, and efficiency.

In contrast to assays reported by others (28, 29), we first quantified the target HPV nucleic acid by generating a standard curve with a known quantity of plasmid DNA from the total cDNA of the target pathogen and host plant. This step could help to stabilize assay sensitivity when performed using plant samples, in which the virus titer is unknown and possibly low. In some diagnostics applications it can be important for users having minimal expertise or training to be able to perform the assay in the field. The battery operated Razor Ex is suitable for detecting several plant pathogens in the field, and results can be obtained in about 25 to 40 min (10, 16). To minimize sample cross-contamination, commercially available Razor Ex pouches filled with lyophilized PCR components eliminate the need for cold storage. In this re-

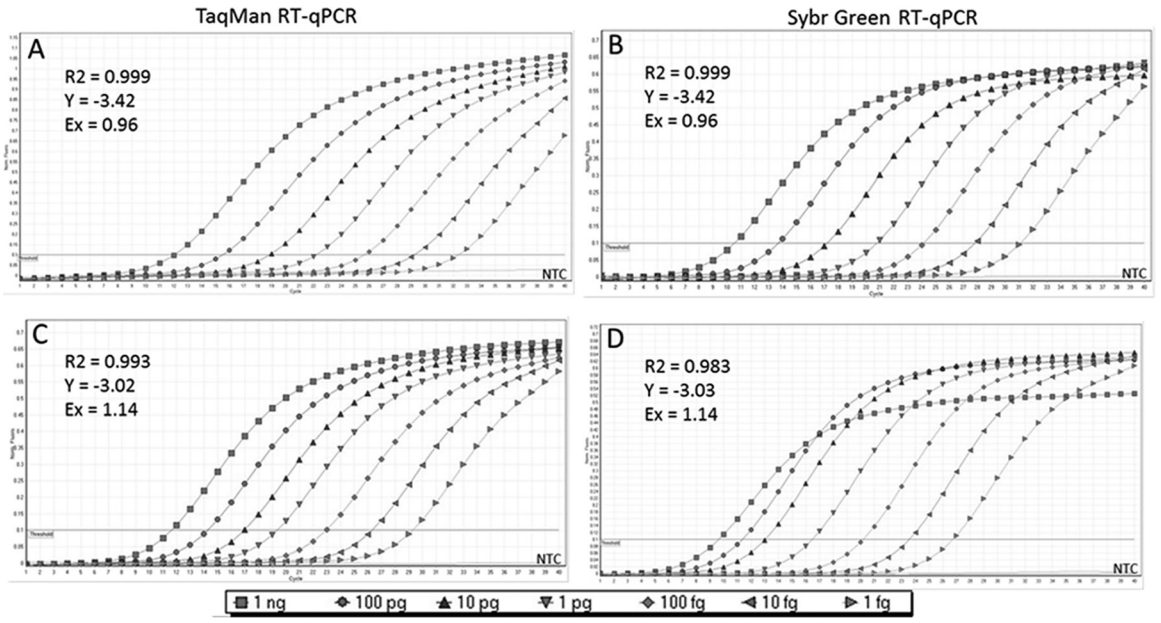

FIG 5 Sensitivity assays using real-time TaqMan RT-qPCR and SYBR green RT-qPCR. Target cDNA from HPV-infected wheat leaves and/or plasmid DNA carrying the target gene fragment was serially diluted in 10-fold increments and used at 1 ng to 1 fg per reaction. (A and C) Sensitivity assays using TaqMan RT-qPCR with infected cDNA (A) and plasmid DNA (C); (B and D) sensitivity assays using SYBR green RT-qPCR with infected cDNA (B) and plasmid DNA (D). R^2 is linear correlation, Y is the slope, and Ex is reaction efficiency. Each reaction was performed in three replicates. The x axis indicates the number of cycles, and the y axis is normalized fluorescence.

TABLE 3 Average C_T values of sensitivity assays using 10-fold serially diluted HPV cDNA or a positive control (plasmid DNA carrying the target sequence) from 1 ng to 1 fg using TaqMan and SYBR green RT-qPCRs

Parameter[a]	Template amt/reaction	C_T (SD)[b]			
		TaqMan RT-qPCR		SYBR green RT-qPCR	
		With cDNA	With plasmid	With cDNA	With plasmid
R^2		0.999	0.993	0.999	0.983
Y		–3.42	–3.02	–3.42	–3.03
Ex		0.96	1.14	0.96	1.14
C_T	1,000 pg	12.08 (0.04)	11.67 (0.05)	10.56 (0.0)*	9.62 (0.05)*
	100 pg	15.51 (0.03)*	14.31 (0.04)	13.71 (0.03)	11.32 (0.06)
	10 pg	19.02 (0.22)	16.73 (0.20)	17.26 (0.15)	12.94 (0.09)
	1 pg	22.52 (0.44)	19.34 (0.02)	20.71 (0.12)	16.40 (0.09)
	100 fg	25.89 (0.24)	23.06 (0.08)	24.33 (0.35)	20.23 (0.02)
	10 fg	29.22 (0.03)	26.47 (0.12)	27.74 (0.13)	23.61 (0.46)
	1 fg	32.58 (0.17)	29.66 (0.37)	30.76 (0.49)	26.73 (0.03)
	NTC[c]	–	–	–	–

[a] R^2, linear correlation; Y, slope; Ex, reaction efficiency. The C_T values are averages of three replications.
[b] Each reaction was performed in three replicates. –, negative (no amplification). *, one replicate was removed from the analysis because of a higher difference in C_T value. SD is standard deviation of three replicates.
[c] NTC, nontemplate control (water).

search, however, we inserted standard TaqMan qPCR reagents into empty pouches by means of a disposable syringe. The Razor Ex sensitivity was not compared; however, others have reported no difference in detection sensitivity between the Razor Ex and the Applied Biosystems 7300/7500 thermocycler formats (16).

Diagnostic and detection protocols may be further enhanced by coupling them with convenient accessory sampling strategies and tools. For example, an elution-independent collection device (30), recently developed for the rapid collection, detection, and storage of plant pathogens, can be used for rapid (3-min) RNA isolation from plant samples and thus can streamline sample preparation.

For in-field diagnostics, isothermal techniques such as RT-HDA offer an inexpensive alternative to PCR machines. However, this technique can be more susceptible to cross-contamination than the other four assays used here (unpublished observations).

Many labs around the world still lack access to thermal cyclers, but a simple and low-cost option could be attractive to extension workers and farmers. Plant disease diagnosticians also may prefer a cost-effective, accurate, and rapid method. We compared the five developed methods for cost, time, accuracy, and facilities and expertise required. We found the Razor Ex method easiest to perform and the most rapid; however, its initial purchase price and per-reaction cost is higher compared to the other methods (Table 2). TaqMan RT-qPCR is more specific than SYBR green RT-qPCR because the SYBR green dye attaches to every double-stranded DNA, including primer dimers, which can result in false-positive results near the end of PCR cycles. However, SYBR green showed higher sensitivity than TaqMan RT-qPCR (Table 3).

The *in vitro* and field-deployable methods reported here for the detection of different variants of HPV are all rapid, reliable, efficient, and sensitive and can facilitate phytosanitary diagnostics and pathogen detection. Each of the five assays has unique characteristics, so comparing their effectiveness and cost allows users to select the combination of features most appropriate for their needs. The Razor Ex system can be used in quarantine locations to prevent pathogen dissemination in plant materials during interstate or international commerce. The assays also have potential applications for monitoring resistance in plant breeding programs and farm management and as models for assay development for other phytopathogens.

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