Title: A rapid isothermal assay for the detection of Hop stunt viroid in hop plants (*Humulus lupulus*), and its application in disease surveys

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Abstract:

Hop stunt disease caused by *Hop stunt viroid* (HSVd) is a growing threat to hop cultivation globally. HSVd spreads mainly by use of contaminated planting material and by mechanical means. Thorough testing of hop yards and removal of infected bines are critical components of efforts to control the spread of the disease. Reverse transcription-polymerase chain reaction (RT-PCR) has become the primary technique used for HSVd detection; however, sample handling and analysis are technically challenging. In this study, a robust reverse transcription-recombinase polymerase assay (RT-RPA) was developed to facilitate analysis of multiple samples. The assay was optimized with all major variants of HSVd from other host species in addition to hop variants. Used in conjunction with sample collection cards, RT-RPA accommodates large sample numbers. Greenhouse and farm samples tested with RT-RPA were also tested with RT-PCR and a 100% correlation between the two techniques was found.

Key words:

Reverse transcription-recombinase polymerase assay; *Hop stunt viroid*; *Humulus lupulus*; Isothermal amplification; FTA sample cards.

1. Introduction

Hop stunt disease was recognized as a major threat to hop production since the first description of the disease in Japan in 1940 ([Yamamoto et al., 1973](#)). Cones of affected hop plants tend to be abnormally small with reduced alpha- and beta-acid contents, the latter characteristics have direct effect on consumer preference ([Yamamoto et al., 1973](#)). In recent years, hop stunt disease has been reported in most major hop growing areas worldwide, and represents a growing threat to hop cultivation globally ([Parkinson and Reed, 2013](#); [O'Neal, 2015](#)). The U.S. is the second largest producer of hops in the world and the largest producer of alpha-acid. In 2015, about 43,633 acres of U.S. land was used for hop cultivation. Washington State alone accounts for 75% of U.S. hop production ([NASS, 2015](#)). In 2004, hop stunt disease was detected in hop yards of Washington State for the first time ([Eastwell and Nelson, 2007](#)).

Hop stunt disease is expressed as severe reductions in bine growth and in yield of certain hop cultivars; the causal agent of this disease was identified as *Hop stunt viroid* (HSVd) ([Sasaki and Shikata, 1977](#)). HSVd is a circular RNA molecule of 296 to 303 bases. The HSVd RNA genome is thermodynamically stable due to secondary structures formed by intermolecular bonding between bases of the viroid, giving it a characteristic rod shaped structure under certain conditions ([Sänger et al., 1976](#)). No vector has been identified for this disease, contaminated plant material has been the major source of disease dispersal ([Pethybridge et al., 2008](#)). In addition to hops, HSVd also infects grapevines, citrus, cucumber, plums, peaches, almonds, apricots and other stone fruits.

HSVd sequence variants are found among these different host species, however categorization of these variants has been inconsistent. Based on sequence and natural hosts, five major

clusters of HSVd isolates have been suggested: plum-peach-almond clade, German grapevine clade, general grapevine-hop clade, U.S. citrus cachexia clade and citrus-cucumber clade (Sano, 2003). Although the degree of pathogenicity of the HSVd variants differ on hop plants, four natural variants isolated from different hosts (HSVd-citrus, HSVd-Plum, HSVd-hop and HSVd-grapevine) were tested and all were able to infect and elicit symptoms in hop plants (Kawaguchi-Ito et al., 2009).

To manage hop stunt disease, robust diagnostic tools are essential. A cucumber bioassay for HSVd was replaced by molecular assay methods including reverse transcription-polymerase chain reaction (RT-PCR) and hybridization assays (Takahashi, 1979; Yaguchi and Takahashi, 1984; Eastwell and Nelson, 2007). These time consuming and technically challenging molecular methods are problematic when faced with large numbers of samples as might be encountered in a survey. An alternative molecular technology, recombinase polymerase amplification (RPA), was developed for detection of DNA sequences (Piepenburg et al., 2006) but was later adapted to the detection of *Phytophthora* spp. (Miles et al., 2015), and various plant pathogenic RNA viruses such as *Little cherry virus 2* (Mekuria et al., 2014), and *Plum pox virus* (Zhang et al., 2014). The isothermal amplification system and easy sample plant sample preparation make this technology potentially very useful for processing and analyzing large numbers of samples. The total time required for the complete test procedure, including sample preparation, is less than 1 hour for each sample relative to 4 hours of time required to complete RT-PCR assay.

2. Materials and methods

2.1. Total RNA isolation

Total RNA was isolated from leaf tissues with the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). A composite pool of five infected or five healthy hop leaves were used for each extraction. Approximately 600 mg tissue was homogenized in a mesh-lined grinding bag (Agdia, Inc., Elkhart, IN) containing 3 mL RLT buffer (Qiagen) amended with 3% 2-mercaptoethanol, 2.5% polyvinylpyrrolidone (PVP-40) and 0.1% sodium metabisulfite. The lysate was incubated at 65°C for 10 min, and centrifuged at 8400 g for 2 min. The supernatant was used for RNA isolation following the kit manufacturers' recommendations. The same procedure was used to isolate RNA from leaves of cucumber, plums, grapes and citrus plants.

2.2. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA samples were tested for the presence of HSVd using RT-PCR in a one-step reaction (SuperScript III one step system with Platinum *Taq* DNA polymerase: Invitrogen, Carlsbad, CA). Primers HSVd1 (5' GCCCCGGGGCTCCTTTCTCAGGTAAG 3') and HSVd2 (5' GGCAACTCTTCTCAGA-ATCC 3') were used for the detection of HSVd ([Eastwell & Nelson, 2007](#)). First strand synthesis was performed at 60°C for 30 min. DNA polymerase activation was done at 94°C for 2 min followed by 35 cycles of 94°C for 15 sec, 59°C for 30 sec and 68°C for 1 min, followed by a final extension step at 68°C for 10 min. Amplification products were resolved using a capillary electrophoresis system (QIAxcel, Qiagen) and product sizes were determined by comparison to a 50-800 bp size marker (Qiagen).

2.3. Fresh sample preparation for RPA

In a mesh-lined grinding bag (Agdia), each fresh leaf samples (total weight approximately 300 mg) was homogenized in 3 mL of general extraction buffer 3 (GEB3, Agdia) containing 2%

Tween-20. The homogenate was used immediately or stored in -20°C. An aliquot of the homogenate (1 µL) was tested for the presence of HSVd in the RT-RPA assay method.

2.4. Sample preparation from sample collection cards

Four leaves per plant were pressed onto Whatman™ FTA™ Plant Saver Cards (Fisher Scientific, Inc., Pittsburgh, PA) following the manufacturers' recommendations. These cards were stored at -20°C until used for nucleic acid extraction. Four discs (2mm) were excised from each FTA card, to which 200 µL of general extraction buffer 3 (GEB3, Agdia) containing 2% Tween-20 was added and incubated overnight at 4°C. One µL of this extract was template for RT-RPA and RT-PCR methods.

2.5. *In vitro* transcription of HSVd RNA and inoculation of cucumber plants

A trimeric construct of HSVd was cloned into pCRII-topo vector (Invitrogen). Sequencing with M13 primers confirmed the presence of the trimeric form of a locally obtained HSVd isolate cloned into the vector. The vector was linearized using high fidelity NcoI restriction enzyme (New England Biolabs, Ipswich, MA). HSVd trimer was transcribed using MEGAscript® T7 Transcription Kit (Life technologies, Carlsbad, CA) following manufacturer's instructions. The purity and yield of RNA transcripts were determined by measuring U.V. absorbance (Genesys 10SUV-vis spectrophotometer; Thermo Fisher Scientific Inc., Waltham, MA).

The primary leaves of germinating cucumber plants (*Cucumis sativus* L. cv. Suvo Long) were inoculated with 10 µL of HSVd trimeric transcripts (2000 ng/µL). The plants were maintained under greenhouse conditions at 28°C with 14 h light and 8 h dark cycles.

2.6. RT-RPA assay

Sequences of nine HSVd isolates from hop were retrieved for analysis ([Sano et al., 2001](#); [Kawaguchi-Ito et al., 2009](#)) ([Fig 1](#)). Using the Bioedit ClustalW tool ([Hall, 1999](#)), multiple sequence alignment of these sequences was performed to identify conserved sequences among different isolates of HSVd. The region between nucleotide positions 56 to 191 of HSVd isolate hKFKi is conserved among all HSVd isolates examined, and was selected for designing primers ([Fig. 1](#)). Selection of primers and probes followed guidelines provided with the RT-RPA system (AmplifyRP® Acceler8® Discovery Kit, Agdia). The probe was 5'-labeled with 6-carboxyfluorescein (FAM) and 3'-labeled with phosphate to act as a blocking agent. An internal 1', 2'-dideoxyribose spacer nucleotide was placed 34 nucleotides from the 5'-terminus of the probe. Reverse primer HSVd R191/160 was labeled with a 5'-biotin moiety. The forward primer HSVd F56/85 was neither blocked nor labeled ([Fig. 1](#)).

Different concentrations of primer-probe combinations were evaluated to provide rapid and reliable detection. Primer concentrations between 105 nM to 840 nM, and probe concentrations between 30 nM to 240 nM were used during standardization of the assay (data not shown). The final conditions adopted for the RT-RPA assay were primer and probe concentrations of 420 nM and 120 nM, respectively, with 0.5 µL Superscript II reverse transcriptase enzyme (200 U/µL, Life technologies) during reverse transcription. After assembly, the reaction mixture was typically incubated for 20 min at 39°C before the amplification products were detected in a lateral flow immunoassay system (Amplicon Detection Chambers, Agdia).

2.7. Sources of hop samples used for validation the assay:

To validate the RT-RPA assay, samples from six different cultivars of hop ('Willamette', 'Nugget', 'Mt. Rainier', 'Chinook', 'Bittergold' and 'Golden star') that tested positive for HSVd by RT-PCR and nine cultivars of hop ('Willamette', 'Nugget', 'Mt. Rainier', 'Chinook', 'Galena', 'Strisslespalter', 'Saaz 72', 'Glacier' and 'Cluster') that tested negative for HSVd were collected from green house and screen house grown plants of the Clean Plant Center Northwest (CPCNW, Washington State University, Prosser, WA). Fresh crude extracts from leaf samples were used as template for RT-RPA assay.

Additionally, 27 samples of seven cultivars ('Cascade', 'Vanguard', 'Galena', 'Apollo', 'Zeus', 'Chinook' and 'Cluster') from five commercial hop yards located in Eastern Washington were collected using FTA cards. Extracts from FTA cards were used as template for both RT-RPA and RT-PCR.

Hop latent viroid (HLVd) is the most prevalent viroid occurring in hop plants ([Pethybridge et al., 2008](#)). To eliminate any chance of cross reactivity of HSVd RT-RPA primers and probe with HLVd, crude extractions from hop plants that tested positive for HLVd by RT-PCR were also tested by the RT-RPA assay.

3. Results

3.1. Optimization of RT-RPA conditions

The optimal primer and probe concentrations for the RT-RPA assay were found to be 420 nM and 120 nM, respectively using 0.5 µL Superscript II reverse transcriptase enzyme (200 U/µL, Life technologies). At 39°C, the reaction period was found to be optimal at 20 min for fresh leaf extracts. Incubation for 10, 15 and 30 min at 39°C, also produced detectable amplification products but appearance of the test band in the lateral flow device was delayed relative to a 20

min incubation time (data not shown). There is little discernable difference in the amplification when reactions were performed at 37°C, 39°C or 42°C. However, when the reaction was conducted at 39°C, appearance of the test band developed quicker than at 37°C or 42°C. Thus, a reaction at 39°C for 20 min was chosen to be optimal for fresh hop leaf extracts for the HSVd primer/probe combination determined in this study. Under the above conditions, a positive reaction was detected in the lateral flow devices within 20 min.

FTA plant saver cards are mainly used for long term storage of nucleic acids on paper matrix (Picard-Meyer et al., 2006). FTA cards used for this study were stored at -20°C for two years after collecting the sample. An extended incubation period of 30 min was selected for amplification for samples from FTA cards to increase the intensity of the bands in positive reactions to make interpretation of results more obvious. However, there was no contradiction in results obtained after 20 min versus 30 min.

3.2. Sensitivity of assays

Different concentrations of total RNA isolated from HSVd-infected plants of hop cultivar Chinook were used as template for the assay to determine the sensitivity of the test. A positive reaction was detected with the lateral flow strip within 20 min when as little as 10 ng/μL of total RNA was used as sample. However, development of the lateral flow strip occurred faster when 100 ng/μL of total RNA was used in the amplification reaction. These results are comparable to the results obtained with RT-PCR (Fig. 2). When a water negative control was used in the RT-RPA assay under the conditions described above, a faint band occasionally appeared after 30 min incubation of the lateral flow strip, suggestive of a positive reaction.

However, this background test band was never detected when crude extracts, FTA card extracts, or when GEB3 buffer were used as the negative control sample.

Sensitivity of this assay was further explored using a HSVd RNA transcript. The *in vitro* transcribed trimeric construct of HSVd was serially diluted in crude sap extract from healthy hop leaves. Crude hop extract was used since RT-RPA can be performed directly in such extracts; the ability to perform the assay without prior purification of RNA is a major advantage of this technology. RT-RPA detected 2×10^9 copies of HSVd transcript diluted in crude extract of healthy plant. The same HSVd transcripts were serially diluted in water for RT-PCR reactions. RT-PCR detected 2×10^4 copies of HSVd trimeric transcript diluted in water. Although RT-PCR provided more sensitivity for HSVd diagnosis, RT-RPA considerably reduced the time of detection, ease and cost effectiveness, thus making it more practical under field conditions for primary diagnosis where using advanced and sensitive techniques is not feasible.

3.3. Validation of RT-RPA assay

HSVd detection for hop samples collected from green houses and screen houses at CPCNW showed complete correlation between results obtained with RT-RPA assays of crude extracts and results obtained by RT-PCR assays of isolated total RNA. For samples collected using FTA cards, all positive reactions in the RT-RPA assay system were evident within 30 min of initiating the lateral flow detection. The results of RT-PCR and RT-RPA were in complete agreement with each other ([Table 1](#)). Despite the lower sensitivity of RT-RPA relative to RT-PCR in terms of the number of HSVd RNA copies detectable, for practical applications, the methods were equally reliable in identifying infected hop plants.

3.4. Specificity of the assay

Sequence analysis of the HSVd transcript used for inoculating cucumber plants revealed that the cloned sequence is identical to HSVd isolate hKFKi (GenBank accession AB039271).

Sequence data of RT-PCR amplicons from several field samples suggested that HSVd isolate hKFKi (GenBank accession: AB039271) represented the predominant HSVd isolate in Washington hop yards ([Eastwell & Nelson, 2007](#)).

At 35 days after the HSVd *in vitro* transcript was used to inoculate cucumber cotyledons, the RT-RPA assay yielded positive reactions for all samples from inoculated cucumber, but none of the non-inoculated control plants. This demonstrated that the positive reaction with HSVd was not limited to hop plants. These results were also verified by RT-PCR. Major genetic variants of HSVd including HSVd-plum, HSVd-citrus and HSVd-grapevine were evaluated by RT-RPA.

Samples of plum and grapevines infected with HSVd-plum and HSVd-grapevines, respectively, as well as HSVd-citrus variants CVd II a and CV II b were tested by RT-RPA. In all cases, RT-RPA detected these variants with no confounding background reactions.

Hop plants that tested positive for HLVd by RT-PCR were tested by the RT-RPA assay. Results showed that the RT-RPA assay results were negative when HLVd alone is present in the sample. There is no cross-reactivity of the RT-RPA assay for HSVd with HLVd, thus further supporting the utility of this assay for HSVd.

4. Discussion

A random sampling of hop plants in the Washington growing areas in 2004 revealed that 15% of hop plants were infected with HSVd ([Eastwell & Nelson, 2007](#)). Although severe symptoms are expressed only in some cultivars resulting in significant yield loss, symptomless infected cultivars act as reservoirs for HSVd providing a source for the further intrusion of disease into

otherwise healthy hop gardens. Immediate removal of infected vines will reduce the sources of viroid inoculum and thus, slow the spread of hop stunt disease in the future.

Detection of HSVd is the first step to the elimination of infected plants. Various techniques are used for HSVd diagnosis. Visual diagnosis of HSVd is difficult as symptoms do not develop within the first years of infection in some cultivars ([Sano, 2003](#); [Kawaguchi-Ito et al., 2009](#)). Moreover, some tolerant cultivars like 'Nugget' do not show any visual symptoms ([Eastwell, unpublished observation](#)) but can act as a reservoir for viroid that can eventually lead to disease spread.

Bioassays can be conducted on some cultivars of cucumber such as 'Suyo' that show symptoms including stunting and leaf curling within 3 to 4 weeks after inoculation with HSVd ([Sasaki et al., 1989](#)). Yet, this bioassay is difficult to apply to testing of large numbers of samples.

Furthermore, results are obtained only after a protracted incubation period after inoculation.

In recent years, hybridization or RT-PCR assays have been the primary techniques used for HSVd diagnosis. These molecular methods accelerated the diagnosis process and increased sensitivity relative to bioassays; this helped restrict HSVd epidemics. However, these methods require technical expertise for sample handling, including the requirement for RNA isolation.

Viroids are very stable at room temperature, and persistent contamination during sample handling is a constant threat during the multiple steps required for RNA isolation, particularly for RT-PCR. To mitigate these concerns, the RT-RPA protocol was developed as an alternative to existing methods, and is amenable to large scale surveys of hop yards. Samples can be processed immediately and the crude extracts used for RT-RPA. Alternatively, samples can be pressed onto FTA cards for subsequent sample analysis utilizing the RT-RPA technology. Both strategies provide a reliable system for viroid diagnosis. During developmental trials, all test

results of hop samples subjected to the RT-RPA assay were substantiated by RT-PCR to confirm the reliability of the test (Table 1 & Fig. 2).

RT-RPA was also tested with four major genetic variants of HSVd. The primers and probe designed for this method were based on conserved regions of all major clusters of HSVd (Fig. 1). RT-RPA assays of crude extracts from hop, grapevine, citrus and plum yielded assay results that reflected the HSVd-infection status of the tissue. These results verified the potential global application of this assay system.

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Fig.1. Multiple sequence alignments of nine *Hop stunt viroid* isolates (GenBank accessions: AB039271, AB039266, AB039268, E00276, AB039269, AB039265, AB039267, AB039270 and AB039272) were used to determine regions of identity. Sequences selected for primers (Forwards primer F56/85: 5' GGTACTTACCTGAGAAAGGAGCCCCGGGGC 3' and reverse primer R191/160: 5' biotin GAAGGTAAAGAAGAAGGGACGATCGATGGTG 3') and probe (/56-FAM/CTT-CTCAGAATCCAGCGAGAGGCGTGGAGAGAGG-/idSp/GCCGCGGTGCTCTGGAG/3Phos) used in the reverse transcription recombinaase polymerase amplification assay are indicated by blue and black boxes, respectively.

Fig.2. Comparison of RT-RPA and RT-PCR assay A). From left to right, reverse transcription recombinase polymerase amplification assay results with total RNA extracted from *Hop stunt viroid*-infected hop plants. Each tube represents amplicon detection chambers (Agdia) with later flow immunoassay strips. CB and TB indicate control band and test band respectively. B).The same total RNA was used as template in reverse transcription polymerase chain reaction assays. The left lane of the gel represents the 50-800 bp marker (Qiagen). The total RNA concentrations in the samples 1 to 5 for each lane are 594 ng/ μ L, 100 ng/ μ L, 50 ng/ μ L, 10 ng/ μ L and 0 ng/ μ L, respectively.

Table 1. Samples collected from commercial fields located in eastern Washington using Whatman™ FTA™ Plant Saver Cards. One microliter of nucleic acid extract from FTA cards was used as template for both Reverse transcription recombinase amplification (RT-RPA) and Reverse transcription polymerase chain reaction (RT-PCR) assays.

Sample	Cultivar	RT-RPA	RT –PCR
FTA 1	Cascade	+ ^a	+
FTA 2	Cascade	+	+
FTA 3	Cascade	+	+
FTA 4	Cascade	+	+
FTA 5	Cascade	+	+
FTA 6	Cascade	+	+
FTA 7	Cascade	+	+
FTA 8	Cascade	+	+
FTA 9	Cascade	+	+
FTA 10	Cascade	–	–
FTA 11	Vanguard	+	+
FTA 12	Vanguard	+	+
FTA 13	Galena	–	–
FTA 17	Apollo	+	+
FTA 18	Apollo	+	+
FTA 19	Apollo	+	+
FTA 21	Zeus	–	–
FTA 2a	Cascade	–	–
FTA 3a	Cascade	+	+
FTA 4a	Cascade	+	+
FTA 5a	Cascade	+	+
FTA 6a	Cascade	+	+
FTA 23	Chinook	+	+
FTA 24	Cluster	+	+
FTA 25	Chinook	+	+
FTA 26	Chinook	–	–
FTA 27	Zeus	–	–

a. (–) = Negative and (+) = Positive reactions.