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Detection and quantification of four viruses in *Prunus* pollen: Implications for biosecurity

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ABSTRACT

Pollen transmitted viruses require accurate detection and identification to minimize the risk of spread through the global import and export of pollen. Therefore in this study we developed RT-qPCR assays for the detection of *Cherry leaf roll virus* (CLRV), *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV), and *Cherry virus A* (CVA), four viruses that infect pollen of *Prunus* species. Assays were designed against alignments of extant sequences, optimized, and specificity was tested against known positive, negative, and non-target controls. An examination of assay sensitivity showed that detection of virus at concentrations as low as 10^1 copies was possible, although 10^2 copies was more consistent. Furthermore, comparison against extant assays showed that in both pollen and plant samples, the newly developed RT-qPCR assays were more sensitive and could detect a greater range of isolates than extant endpoint RT-PCR and ELISA assays. Use of updated assays will improve biosecurity protocols as well as the study of viruses infecting pollen.

1. Introduction

Pollen is a vital commodity for both plant breeding, and commercial fertilization of many field and horticultural crops. It is also a means by which some plant viruses are transmitted between hosts. Pollen transmission may be vertical, through the germ line into the next generation of plants, or horizontal, via the fertilized flower and subsequent systemic infection of an adult plant (Jones, 2018). Besides being a means of transmission, virus presence in pollen has also been shown to reduce pollen viability and fertilization rates (Jones, 2018)

The worldwide commercial movement of pollen into new growing regions or countries presents a biosecurity risk for national plant protection organizations (NPPOs). This risk can be managed or mitigated by diagnostic screening of pollen samples, but as with any regulatory requirement, the effectiveness of such a regime is reliant on the specificity and sensitivity of the diagnostic methods applied.

The four viruses examined were *Cherry leaf roll virus* (CLRV), *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV), and *Cherry virus A* (CVA). The first, CLRV, is a nepovirus with a broad host range beyond *Prunus*, including both herbaceous and woody perennials (Rebensdorf et al. 2006). CLRV is readily transmitted vertically both through infected seed or pollen; horizontal transmission via pollen appears much less effective (Massalski and Cooper, 1984). PDV and PNRSV are both ilarviruses that primarily infect *Prunus* species, although PNRSV has also been found in crops as diverse as roses, apples and hops (Hammond and Crosslin, 1998). Both vertical and horizontal transmission has been reported from these two viruses (Silva et al., 2003; Card et al., 2007), which may help explain their near-ubiquity in stonefruit producing areas (Harper, unpublished). Finally, CVA, a capillovirus only known to infect cherries was included as it has no known invertebrate vector, is extremely common in commercial cherries, and is seed transmitted at high frequency (Harper, unpublished).

Therefore, in this study we developed and optimized methodologies for the extraction and RT-qPCR detection of four viruses that infect pollen of *Prunus* species (cherries, peaches, etc), and compared and contrasted to extant methods currently used or required by NPPOs. We found that all four viruses could be readily detected in *Prunus* pollen by RT-qPCR, although the extraction procedure and RNA quality played a significant role in accurate detection. Virus titer varied considerably between the four species, influencing detection by less sensitive methods, and interestingly was not significantly lower in pollen than in infected plant tissue. These findings will aid in the development and implementation of pollen screening regimes for both breeding programs and NPPOs.

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2. Methods

2.1. Sample collection and extraction

Plant samples were obtained from the collection maintained at the Washington State University's Irrigated Research and Extension Center in Prosser, WA. Total RNA was extracted from all samples using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), with the addition of 2.5% w/v PVP-40, 0.1% v/v sodium metabisulfite (Siju et al., 2007), and 0.2% v/v Antifoam A to the extraction buffer.

Pollen samples were obtained from a range of commercial sources in central Washington, as well as from the WSU-IAREC collection. Total RNA extraction was as above, with modification of different factors assessed for optimization purposes. These were: pollen input mass (10 mg–100 mg in a 10 mg increment); modified (additional 2.5% w/v PVP-40, 0.1% v/v sodium metabisulfite, and 0.2% v/v Antifoam A) vs. unmodified RLT buffer; Maceration using a Homex6 homogenizer (Bioreba) vs. bead-beating in Tissuelyzer (Qiagen) with 6 mm steel beads, or less disruptive incubation at 65 °C for 10 min, either with or without vortex disruption every 2 min.

2.2. RT-qPCR primer design

The primers and TaqMan probes for RT-qPCR amplification of the four target viruses (Table 1) were designed using the Biosearch Real-Time Design online software (LGC Biosearch Technologies, Novato, CA) against an alignment of representative complete genome sequences obtained from the NCBI database were followed for each virus (listed in Supplementary Table 1). Primer and probe properties, including T_m, self- and hetero-dimer formation were performed *in silico* using Oligo Analyzer online software (IDT Technologies, Coralville, IA).

2.3. RT-qPCR reaction optimization

The reaction conditions for each of the designed primer and probe sets were optimized on a BioRad CFX96 real-time thermocycler (BioRad Laboratories, Hercules, CA) using Invitrogen SuperScript™ III Platinum™ One-Step qRT-PCR reagents (Thermo-Fisher Scientific, Waltham, MA). Each method was optimized against known positive, negative, and no-template controls. Positive and negative samples were confirmed for CLRV, CVA, PDV, and PNRSV using extant endpoint RT-PCR assays currently in use (respectively, K. Eastwell, WSU *unpublished*; Marais et al., 2011; Rampitsch et al., 1995; Vaskova et al. (2000). The reaction conditions for temperature (57 °C–70 °C), $MgSO_4$ concentration (3 mM–6 mM), primer concentration (300 nM – 600 nM), and probe concentration (50 nM - 200 nM) were also examined.

2.4. Reaction specificity, sensitivity, and comparison to extant assays

Assay specificity was assessed against a panel of known positive samples for each virus, identified by other extant assays and/or high-throughput sequencing (data not shown), against non-targtet viruses from the same genera or frequently found in each host, and against virus-free representative hosts from *Prunus avium, P. persica, P. domestica,* and related species to ensure the reactions did not cross-react with the virus hosts.

To determine assay sensitivity and identify the limit of detection, synthetic transcripts spanning the respective of the CLRV, CVA, PDV, and PNRSV target regions were produced via by RT-PCR using virus-specific primers (Table 1) as per (Beaver-Kanuya et al., 2019). Serial dilutions from 10^9-10^1 copies per µl were prepared in virus-free total RNA extracts from cherry to better reflect the effects of inhibitors in plant tissues. Each dilution series point was tested in triplicate to account for technical error, and the reaction efficiency was calculated using CFX96 manager software (BioRad Laboratories, Hercules, CA).

Finally, the assays developed in this study were compared to extant endpoint RT-PCR assays (K. Eastwell *unpublished*; Marais et al., 2011; Rampitsch et al., 1995; Vaskova et al. (2000), for CLRV, CVA, PDV, and PNRSV respectively) against a panel of greenhouse, field, and pollen samples collected from known and unknown positive and negative samples. The CLRV, PDV, and PNRSV assays were also compared to ELISA assays (Agdia Inc., Elkhart, IN), performed as per the manufacturer's instructions.

3. Results and discussion

We began this study by developing RT-qPCR assays for the detection of our four target pathogens, PDV, PNRSV, CLRV, and CVA. Though the optimization process (refer to supplementary Table 1) we found many core conditions remained the same, regardless of assay, these being

Table 1

Primers and probes developed in this study for the amplification of *Cherry leaf roll virus* (CLRV), *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV), and *Cherry virus A* (CVA) by RT-qPCR and for generating the RNA transcripts.

Assay	Primer Name	Orientation	Sequence (5'-3')	Binding Site
CLRV RT-qPCR	CLRV-RT-F	+	CCATCCATGGCCGGTTAGTC	6105-6128
	CLRV-RT-R	–	CGGCACATTCATGCCACATG	6160-6179
	CLRV-RT-P	+	6FAM- TCGGTTCTGGACTAGTGCAAGTGGA -BHQ1	6130-6151
CVA RT-qPCR	CVA-1-F	+	CGTGCGAAGGACATTTACAGTG	5475-5496
	CVA-1-R	–	CCAGTGCAGCTTGCAGGTATAG	5557-5578
	CVA-1-P	+	6FAM- TGCAAATGCCTTCAATTCCAAAGTGC -BHQ1	5498-5523
PDV RT-qPCR	PDV-1-F	+	CACAGCGAAACAAGCCAGTG	1614-1633
	PDV-1-R	–	GCATCGAGTGTTGGAGGTACTG	1750-1771
	PDV-1-P	+	6FAM- TCTTGCTCCTACTGGTATGACCGTTG -BHQ1	1644-1669
PNRSV RT-qPCR	PNRSV-1-F	+	GGGATGGTGGAGGACTATAAGG	1505-1526
	PNRSV-1-R	–	CTCTCGGTTGGTCCTTCAAGA	1569-1589
	PNRSV-1-P	+	6FAM- TGGATCAACCTGATGGTCCGAATGC -BHQ1	1526-1551
CLRV RNA Standard	CLRV-S-T7-F	+	TAATACGACTCACTATAGGG CGAATATCTACTGCCATCCGTA	6070-6094
	CLRV-S-R	–	CCGACACTCATACAATAAGCGAAT	6259-6282
CVA RNA Standard	CVA-S-T7-F	+	TAATACGACTCACTATAGGGGTTAGAAGGTCACGCTCAAT	5471-5490
	CVA-S-R	–	GCTCCAAGATGAAGCATTGA	5652-5671
PDV RNA Standard	PDV-S-T7-F	+	TAATACGACTCACTATAGGGG TTTCGATGTGAGTGCTCCTG	1563-1582
	PDV-S-R	–	TCGCATCTATGAAAGCGGTA	1818-1837
PNRSV RNA Standard	PNRSV-S-T7-F	+	TAATACGACTCACTATAGGGG TCCCTCAGTTGATGGGTCAG	1425-1444
	APNRSV-S-R	–	GAAATCTAAATCGGAGGGAGGT	1603-1624

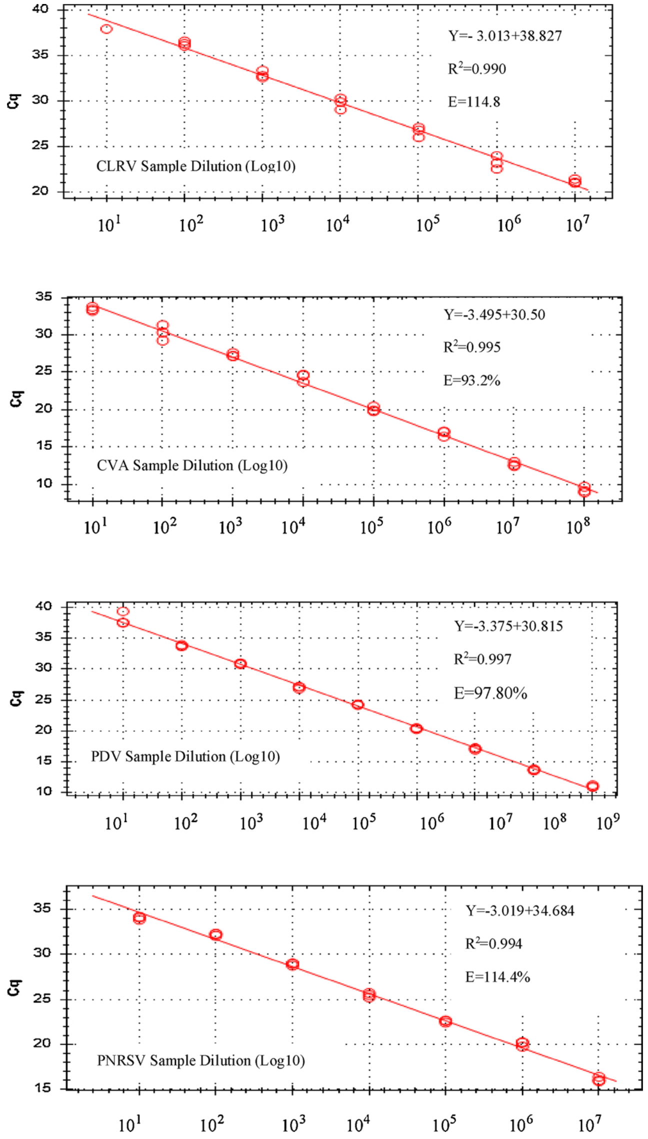

Fig. 1. Standard curves generated from the serial dilution (10^9 through 10^1 copies per reaction) of artificial RNA standards for *Cherry leaf roll virus* (CLRV), *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV), and *Cherry virus A* (CVA). RNA standards were diluted in virus-free total RNA extracts from cherry. Reactions were performed technical replicates of three.

400 nM of sense and antisense primers, 100 nM probe, 0.4 µl of Superscript III/Platinum Taq enzyme mix, in a final volume of 20 µl. Optimal magnesium concentrated differed, with CLRV, PDV, and PNRSV assays requiring a final concentration of 6 mM MgSO4, whilst CVA showed no significant change and so remained at the base 3 mM level. Reactions conditions were optimized to the same temperature regime for each reaction, with reverse-transcription at 50 °C for 15 min, followed by 2 min heat activation/inactivation step at 94 °C, then 40 cycles of 94 °C for 15 s, and 60 °C for 45 s.

Having optimized the assays we then examined their sensitivity and specificity. Examination of sensitivity against artificially generated standard curve diluted in virus-free *P. avium* extract. Each assay was capable of reliably detecting 10^2 copies of their respective target templates per reaction (Fig. 1); detection down to 10^1 copies was possible although technical replicate variation did increase at that low concentration. Specificity was assessed against common host species of the four viruses tested, against each other and other viral species often found in mixture in stone fruit species, and finally against a panel of known and suspected virus-positive cherry samples maintained at WSU's Prosser experiment station. For all four assays developed, no host-reactivity or cross-reactivity with other viruses was observed (Table 2), and detected virus positives in samples that screened negative by the respective species-specific endpoint PCRs (Table 2), which, given the high Cq values generated from those samples, suggests that the new RT-qPCR assays are more sensitive.

Next, using the new RT-qPCR assays we examined and optimized a total RNA extraction procedure for pollen samples. Comparison of grinding using a Homex6 homogenizer (Bioreba), versus bead beating with a Tissuelyzer (Qiagen), versus simple incubating the samples at 65 °C with or without vortexing, all followed by extraction using RNeasy plant minispin columns (Qiagen, as per manufacturer's instructions) was inconclusive (Supplementary Table 2). In contrast modification of the default extraction buffer with 2.5% w/v PVP-40 (Qiagen, manufacturer's recommended modification), 0.1% v/v sodium metabisulfite (Siju et al., 2007), and 0.2% v/v Antifoam A increased sensitivity of detection by approximately 1–13 cycles, depending on extraction method. We also examined the effect of different starting volumes of pollen, and found that as little as 10 mg was sufficient for reliable detection of all four viruses; with a 2-cycle decrease in sensitivity from using 100 mg (Supplementary Table 2).

We then compared detection efficacy and sensitivity of known or suspected virus-positive pollen samples to endpoint RT-PCR and where applicable, ELISA (Table 3). We found that given the generally high titer of the four viruses in pollen, with the possible exception of CLRV, detection sensitivity between endpoint and RT-PCRs was comparable. ELISA however, was less so, missing two CLRV positive, one PDV positive, and four PNRSV positive sample; detection of CVA by ELISA was not examined as there is no commercially available antisera for this virus. Interestingly, the inability of the PNRSV antisera to detect virus in some samples cannot be attributed to virus titer, as Cq values from the same samples indicated a titer comparable to samples that were detected as positive (Table 3). This may indicate that there are variants of PNRSV missed by the monoclonal Agdia antisera. Finally, we examined a further series of pollen samples to examine virus and copy number for the four viruses. As can be seen in Table 4, titer in 100 mg of pollen varied considerably between species. CLRV titer was very low or undetectable in most samples, with copy numbers in the 10^2 to 10^3 range. This is consistent with the low CLRV titer in somatic tissues of woody species, including *Prunus* (Rombou et al., 2009), which, combined with the non-homogenous distribution of CLRV (Büttner et al., 2011), may also account for the low number of samples found to be CLRV positive in this study.

CVA and PNRSV titers were broadly comparable, with approximately 10^2-10^5 copies detected, whereas PDV titer was uniformly high with between 10^6 through 10^8 copies per 100 mg. This is consistent with earlier studies in which titer of both PDV and PNRSV was found to be high within pollen grains from *Prunus* species (Kelley and Cameron, 1986; Aparicio et al., 1999). Interestingly, this study represents the first report of CVA in *Prunus* pollen. In the absence of a known vector species, it had previously been speculated that pollen transmission may occur (Glasa et al., 2017). While presence of CVA pollen is not confirmation of pollen transmission, it is a necessary precondition, it also should be considered that CVA is seed transmissible with rates of up to 80% from single infected trees (Harper, unpublished), and one could speculate that the ovule may become infected through the male germ line.

Pollen represents a major route by which viruses may move from field to field, or country to country, although biosecurity regimes must take into account that detection in pollen is not prima facie evidence of

Table 2
Comparison of the RT-qPCR assays designed in this study to extant endpoint RT-PCR and ELISA assays for the detection of *Cherry leaf roll virus* (CLRV), *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV), and *Cherry virus A* (CVA). Virus detected = +, virus not detected = -.

a)	Species	Cultivar	Sample No.	CLRV		CVA		PDV		PNRSV	
				RT-qPCR Cq	Endpoint RT-PCR	RT-qPCR Cq	Endpoint RT-PCR	RT-qPCR Cq	Endpoint RT-PCR	RT-qPCR Cq	Endpoint RT-PCR
	P. avium	Lapin	1	–	–	24.1	+	17.7	+	19.6	+
			2	–	–	17.3	+	17.7	+	24.1	+
			3	–	–	26.2	+	15.4	+	26.1	+
		Sweetheart	–	27.9	+	22.6	+	18.5	+	–	–
		Gisela-6	–	35.7	+	21.1	+	20.9	+	17.3	+
		Rainier	–	27.1	+	23.8	+	16.2	+	27.9	+
		Bing	1	29.7	+	27.6	+	19.5	+	20.9	+
			2	26.8	+	25.1	+	–	–	16.4	+
			3	27	+	24.9	+	35.2	–	15	+
			4	–	–	35.9	–	36.9	–	35.4	–
			5	–	–	25.3	+	–	–	–	–
			6	32.27	–	14.8	+	13.6	+	22.4	+
		Skeena	–	–	–	21.5	+	17.9	+	23.6	+
		Chelan	1	–	–	21.8	+	15.5	+	37.7	–
			2	–	–	–	–	17.2	+	35.2	–
			3	–	–	24.4	+	17.3	+	33.9	–
		Penny	–	–	–	26.8	–	17.8	+	27.3	+
b)	*P. avium*			–	N/A	–	N/A	–	N/A	–	N/A
	P. persica			–	N/A	–	N/A	–	N/A	–	N/A
	P. domestica			–	N/A	–	N/A	–	N/A	–	N/A
	Humulus lupulus			–	N/A	–	N/A	–	N/A	–	N/A
c)	*Apple stem grooving virus*			–	N/A	–	N/A	–	N/A	–	N/A
	Cherry virus A			–	N/A	24.2	N/A	–	N/A	–	N/A
	Cherry leaf roll virus			32.5	N/A	–	N/A	–	N/A	–	N/A
	Prune dwarf virus			–	N/A	–	N/A	16.6	N/A	–	N/A
	Prunus necrotic ringspot virus			–	N/A	–	N/A	–	N/A	21.2	N/A
	Tobacco ringspot virus			–	N/A	–	N/A	–	N/A	–	N/A

Table 3
Detection efficacy and sensitivity of known or suspected virus-positive pollen samples using the real-time RT-qPCR assays for CLRV, PDV, PNRSV, and CVA, compared to RT-PCR and ELISA assays. Virus detected = +, virus not detected = -.

Cultivar		CLRV			CVA			PDV			PNRSV		
	Sample No.	RT-qPCR Cq	Endpoint RT-PCR	ELISA	RT-qPCR Cq	Endpoint RT-PCR	ELISA	RT-qPCR Cq	Endpoint RT-PCR	ELISA	RT-qPCR Cq	Endpoint RT-PCR	ELISA
Coral	–	31.1	+	+	22.1	+	N/A	17.0	+	+	21.6	+	+
Chinook	–	–	–	–	27.1	+	N/A	17.5	+	+	17.9	+	+
Rainier	1	30.2	+	+	25.2	+	N/A	17.8	+	+	–	–	–
	2	34.3	+	–	23.4	+	N/A	20.9	+	+	27.4	+	+
	3	31.5	+	+	24.1	+	N/A	20.9	+	+	10.3	+	+
	4	31.0	+	+	22.5	+	N/A	22.9	+	+	29.7	+	+
Lapin	1	–	–	–	24.1	+	N/A	17.7	+	+	18.4	+	+
	2	–	–	–	17.3	+	N/A	17.7	+	+	19.6	+	+
	3	–	–	–	26.2	+	N/A	15.4	+	+	24.0	+	–
	4	–	–	–	28.9	+	N/A	23.0	+	+	26.0	+	–
	5	–	–	–	22.3	+	N/A	20.1	+	+	25.9	+	+
Unknown	1	30.0	+	–	18.4	+	N/A	17.7	+	+	30.9	+	–
	2	32.5	+	+	22.1	+	N/A	18.3	+	+	17.2	+	–
	3	35.5	+	+	18.3	+	N/A	19.3	+	+	28.4	+	+
	4	29.5	+	+	21.2	+	N/A	17.3	+	+	33.3	+	+
	5	31.7	+	+	24.9	+	N/A	31.2	+	–	33.4	+	+

transmission potential, as some viruses may be transmitted only through fertilization via the germ tube, whilst others may occur via mechanical damage and transfer during insect pollination (Jones, 2018; Shiller et al., 2010); in the case of the former the need for successful fertilization reduces the likelihood of cross-species transmission (Rebenstorf et al., 2006). The

RT-qPCR assays developed in this study present a useful tool for the further study of four *Prunus*-infecting viruses in both plant somatic tissues and pollen. These assays are both sensitive and robust, and are suitable for use in a biosecurity or laboratory screening environment.

Table 4
Examination of pollen samples to determine virus copy number using the real-time RT-qPCR assays for CLRV, PDV, PNRSV, and CVA.

Cultivar	No. samples	CLRV		CVA		PDV		PNRSV	
		Avg. Cq	Copy Number Range	Avg. Cq	Copy Number Range	Avg. Cq	Copy Number Range	Avg. Cq	Copy Number Range
Bing	4	–	–	19.2 ± 0.2	5.8×10^4 - 1.3×10^5	15.3 ± 2.0	1.2×10^6 - 7.4×10^8	31.5 ± 0.3	4.6×10^1 - 7.5×10^1
Chelan	4	34.74 ± 0.0	9.8×10^2	26.5 ± 3.2	6×10^0 - 9.1×10^4	14.7 ± 5.2	7.8×10^2 - 1.9×10^9	24.9 ± 3.9	3.2×10^1 - 9.6×10^5
Coral Champagne	3	–	–	–	–	12.0 ± 0.5	1.0×10^8 - 3.6×10^8	23.3 ± 0.4	2.0×10^4 - 5.1×10^4
Rainier	11	33.8 ± 1.9	1.4×10^2 - 2.7×10^4	24.7 ± 1.3	1.4×10^1 - 1.5×10^4	12.6 ± 0.8	8.3×10^6 - 1.6×10^8	24.4 ± 1.4	1.3×10^2 - 7.1×10^6
Santina	2	34.96 ± 0.0	8.2×10^2	21.9 ± 0.1	1.3×10^4 - 1.6×10^4	15.3 ± 0.5	1.4×10^7 - 2.6×10^7	28.2 ± 0.3	5.6×10^2 - 8.4×10^2
Skeena	7	–	–	24.9 ± 1.8	1.2×10^1 - 1.1×10^5	22.4 ± 2.6	1.6×10^2 - 7.4×10^7	28.7 ± 2.3	1.1×10^3 - 9.5×10^4
Sweetheart	5	34.18 ± 0.0	1.5×10^3	21.1 ± 0.7	1.7×10^3 - 6.8×10^4	12.6 ± 1.0	8.4×10^6 - 3.9×10^8	19.9 ± 1.9	4.1×10^3 - 7.8×10^6

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.113673.

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