Quantitative Molecular Detection of *Xanthomonas hortorum* pv. *carotae* in Carrot Seed Before and After Hot-Water Treatment

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Abstract

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Molecular assays to detect and quantify DNA from viable cells of the seedborne pathogen *Xanthomonas hortorum* pv. *carotae* in carrot seed were developed and evaluated for use on nontreated and hot-water-treated seed lots. Both a TaqMan real-time polymerase chain reaction (PCR) assay and a loop-mediated isothermal amplification (LAMP) dilution endpoint assay detected and quantified DNA from viable pathogen cells after treatment of carrot seed washes with the live-dead discriminating dye propidium monoazide (PMA). The detection limits of the assays were approximately 10^1 CFU for pure cultures of *X. hortorum* pv. *carotae*, and 10^2 to 10^3 CFU/g seed from naturally infested carrot seed lots. *X. hortorum* pv. *carotae* in and on carrot seed was killed by soaking the seed in hot water (52°C for 25 min), and a subsequent PMA treatment of these hot-water-treated seed washes suppressed detection of the pathogen with both the real-time PCR and LAMP assays. For 36 commercial seed lots treated with PMA but not hot water, regression of colony counts of *X. hortorum* pv. *carotae* measured by dilution plating on a semiselective agar medium versus estimates of pathogen CFU determined by the molecular assays resulted in significant ($P \leq 0.05$) linear relationships ($R^2 = 0.68$ for the real-time PCR assay and 0.79 for the LAMP assay). The molecular assays provided quantitative estimates of *X. hortorum* pv. *carotae* infestations in carrot seed lots in <24 h, which is a significant improvement over the 7 to 14 days required to obtain results from the traditional dilution-plating assay.

The seedborne bacterial pathogen *Xanthomonas hortorum* pv. *carotae* is a common contaminant in and on carrot seed produced in semiarid areas of the Pacific Northwest region of the United States (5). This region supplies 50 to 75% of the U.S. carrot seed market and up to 50% of the seed supply for international markets (5,29). Carrot seed infested with *X. hortorum* pv. *carotae* can serve as primary inoculum for bacterial blight when fresh and processing carrot crops are grown in warm and humid climates (1,5,16,24). Even when bacterial blight symptoms are not observed in carrot seed crops, the seed can become infested with *X. hortorum* pv. *carotae* from asymptomatic, epiphytic populations of the pathogen that reside on leaves and umbels (5). A field study (31) identified a threshold for *X. hortorum* pv. *carotae* contamination of 10^4 to 10^5 CFU/g seed, above which bacterial blight is likely to develop in a fresh-market carrot crop in the warm, semiarid conditions typical of the Central Valley of California. *X. hortorum* pv. *carotae* infestations in commercial carrot seed frequently exceed 1×10^6 CFU/g seed (5); therefore, seed lots are tested routinely for the pathogen (1,6), and lots above the threshold are subjected to hot-water treatment (typically 52°C for 25 min) (5–7).

Traditionally, quantitative measurement of *X. hortorum* pv. *carotae* infestations in carrot seed involves plating a dilution series of a seed wash onto a semiselective agar medium (1,6,13,25,33). This assay requires approximately a week to complete, and is commonly performed a second time after hot-water treatment to assess treatment efficacy. Meng et al. (16) developed a polymerase chain reaction (PCR) assay that detects *X. hortorum* pv. *carotae* DNA at a limit of sensitivity similar to seed wash dilution plating assays (approximately 10^2 CFU/ml seed wash). The seed industry has not, however, deployed the PCR assay to quantify *X. hortorum* pv. *carotae* in carrot seed because the assay cannot differentiate DNA from live and dead pathogen cells, and the assay is not quantitative (1,6,16). A molecular assay that could quantify DNA from only viable *X. hortorum* pv. *carotae* cells would be valuable for testing carrot seed lots, particularly following treatment with hot water.

Recent studies on foodborne bacterial pathogens have demonstrated the potential of a DNA-intercalating dye, propidium monoazide (PMA), for selective detection of DNA from only viable bacterial cells (2,19,20,23). Differentiation is based on the ability of live cells (i.e., those with a functional plasma membrane) to exclude PMA. In contrast, PMA readily penetrates into dead or otherwise membrane-compromised cells. Inside the cell, PMA crosslinks covalently to DNA by exposure to bright light, which renders the bound DNA insoluble; the light treatment also inactivates excess PMA in solution (19). A subsequent genomic DNA extraction from the PMA-treated cell suspension yields DNA from live cells only (19), which then can be subjected to a molecular amplification protocol.

Real-time PCR assays have been developed to quantify DNA from plant pathogens (4,14,18,22,27,30,32), and are a logical choice for assessing *X. hortorum* pv. *carotae* infestation levels in carrot seed. Potential limitations to the use of real-time PCR assays for pathogen detection are the relatively high costs of the real-time thermal cycler and the technical staff, as well as the common problem of PCR inhibitors in environmental samples (15,26,32). Loop-mediated isothermal amplification (LAMP) is an alternative method for amplifying DNA that requires only a heat block or water bath set at 65°C (3,17,21,28). Instruments for real-time LAMP assays have been developed (9,17) but the assay procedure is sufficiently inexpensive that quantification also can be accomplished by a dilution endpoint analysis of extracted DNA. Like PCR assays, a LAMP assay requires only a few hours to complete and LAMP assays are reportedly less sensitive to inhibitors in environmental samples compared with PCR assays (11,17).

The overall objective of this study was to develop molecular assays that quantify DNA from live *X. hortorum* pv. *carotae* cells. To meet this objective, we evaluated a protocol of treating carrot seed

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washes with PMA to eliminate DNA from nonviable (dead) *X. hortorum* pv. *carotae* cells without affecting live cells of the target pathogen. Second, we developed and compared a real-time PCR assay and a LAMP dilution endpoint assay for the quantification of *X. hortorum* pv. *carotae* DNA. Finally, we combined PMA treatment with the two methods of DNA amplification to quantify viable *X. hortorum* pv. *carotae* cells in commercial carrot seed lots before and after hot-water treatment.

Materials and Methods

Bacterial strains and DNA extraction. *X. hortorum* pv. *carotae* strains M081^R and Car111 were used for molecular assay development at Oregon State University (OSU) in Corvallis and at the Washington State University (WSU) Northwestern Washington Research and Extension Center in Mount Vernon, respectively. M081^R is a spontaneous, rifampicin-resistant selection of *X. hortorum* pv. *carotae* strain M081, which was obtained from a carrot seed crop grown near Madras, OR (12), and *X. hortorum* pv. *carotae* Car111 is an isolate from a carrot seed crop harvested in central Washington (5). *X. hortorum* pv. *carotae* strains were cultured and maintained on medium 523 agar (10) or on XCS agar, a semiselective medium for *Xanthomonas* spp. (6,31).

For development of the real-time PCR assay, *X. hortorum* pv. *carotae* genomic DNA was extracted from cell suspensions of the bacterium using the Qiagen DNeasy blood and tissue kit (Qiagen) following the standard protocol for gram negative bacteria, except that incubation time in the final elution step was increased from 2 to 5 minutes to improve DNA yield (Y. Pan, *personal communication*). For the LAMP assay, the same Qiagen DNeasy blood and tissue kit and the InstaGene matrix kit (Bio-Rad Inc.) were used following the standard protocol for bacteria. DNA extraction with the InstaGene matrix was followed with an additional centrifugation of the supernatant (1 min at 16,000 × *g*) through a mini-elute column (Epoch Biolabs Inc.).

Primer selection and evaluation of specificity. For the real-time PCR assay, primers were designed from a region of genomic DNA that was shown to be unique to *X. hortorum* pv. *carotae* based on comparison with the National Center for Biotechnology Information nucleotide database; this 2-kb region of DNA included and flanked the genomic sequence targeted by PCR primer pair XhcPP02 described by Kimbrel et al. (12). ABI Primer Express software (V3.0; Life Technologies Corp.) was used to design the primers and the TaqMan probe (Table 1). For the LAMP assay, the primer set used for this study was based on the sequence of the PCR product of the 9B primer set designed by Meng et al. (16). The amplicon of the PCR product generated with the 9B primers was purified from an agarose gel with a Qiagen Gel Purification Kit (Qiagen), and sequencing of the amplicon was done at the OSU Center for Genome Research and Biocomputing (Corvallis). LAMP primers for this sequence were designed with Primer Explorer software (V4; Eiken Chemical Co.) (Table 1).

Candidate real-time PCR and LAMP primer sets were evaluated for ability to amplify *X. hortorum pv. carotae* DNA from pure cultures, and for specificity to *X. hortorum* pv. *carotae* by testing the primers against DNA from pure cultures of *X. arboricola* pv. *pruni*, *X. axonopodis* pv. *allii, X. campestris* pv. *campestris*, *X. campestris* pv. *vesicatoria*, *X. campestris* pv. *coriandri*, *X. campestris* pv. *phaseoli, X. hortorum* pv. *pelargonii*, two *Bacillus* spp., *Pantoea agglomerans,* four *Pseudomonas* spp., and 14 additional bacteria (see the supplement to the online version of Kimbrel et al. [12] for a list of other bacteria). After determining no cross reaction with DNA from these other bacteria, selected primers were verified by separating amplicons with gel electrophoresis, followed by purification and sequencing as described above. Primer sets were also verified for positive reactions to six additional *X. hortorum* pv. *carotae* strains from carrot seed grown in the United States, Argentina, Chile, and France (12).

Real-time PCR assay and LAMP assay conditions. A TaqMan real-time PCR assay (8) for *X. hortorum* pv. *carotae* was developed using the TaqMan Environmental Master Mix 2.0 (1× concentration) (Life Technologies) in 25-µl reaction volumes under the conditions of an initial 10-min denaturation at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Primer concentrations were 0.4 µM, the probe concentration was 0.2 µM, and 5 µl of DNA extract was used as template. Reactions were performed on a Bio-Rad CFX96 real-time thermocycler (Bio-Rad).

A LAMP assay using turbidity (32) for dilution endpoint detection of *X. hortorum* pv. *carotae* was performed in a hot-water bath or in a real-time turbidimeter (Eiken Chemical) for 1 h at 65°C in 50-µl reactions. Primer concentrations were 2.4 µM each of Fip and Bip, 0.2 µM each of F and B, and 0.4 µM loop F. The master-mix consisted of primers and 1.4 mM dNTPs, 0.8 M betaine, 1× ThermoPol buffer (New England BioLabs), 8 mM $MgSO_4$, 5.0% bovine serum albumen, and 2 µl of *Bst* DNA polymerase (New England BioLabs). DNA extract (10 µl) was used as template.

Standard curves and sensitivity of the real-time PCR and LAMP assays. *X. hortorum* pv. *carotae* strains M081^R or Car111 were grown overnight (16 to 18 h) in medium 523 broth (10) to obtain cultures in log phase. To relate the number of *X. hortorum* pv. *carotae* CFU to concentration of extracted DNA, triplicate suspensions of strains M081^R and Car111 at 1×10^6, 1×10^7, 1×10^8, and 1×10^9 CFU/ml in sterilized distilled water were subjected to DNA extraction as described above, and the yield of DNA was quantified using a Nanodrop spectrophotometer (Corvallis; Thermo Fisher Scientific) or Qubit fluorometer (Mount Vernon; Life Technologies). To evaluate assay sensitivity and develop a standard curve of cycle threshold (C_t) values for the real-time PCR assay or dilution endpoint for the LAMP assay versus the $\log_{10}$ (DNA concentration), a 10-fold dilution series of genomic DNA, ranging from 0.1 fg/µl to 10 ng/µl, was generated in nuclease-free water. Triplicate samples of each dilution were subjected to the real-time PCR and LAMP protocols.

Table 1. Primer sets used for DNA amplification of *Xanthomonas hortorum* pv. *carotae*

Molecular assay and primers	Sequence (5′ to 3′)
PCR (Xhc-q2 primer set)[a]	
F	GCATGAAGGCAATACAGCG
B	CGATCCAGCTGATGTTCTCCGAA
TaqMan probe	56-FAM/TCAAGCTCAGACGAAACCGGCGTC/3BHQ_1
LAMP (Lace primer set)[b]	
Fip	AGAGCAGGCAACTCCGTTCAATACGACTTGAGTCTTGGTGTC
Bip	ACATCATCTGGGCAGGTCCATCGAATCCGCAATTCCCCGTTA
F	CAGCGTCTTCTAAGGTCACC
B	GCTTCAAGTACCGATCACGT
Loop F	CGTGATCTTTCGGACCCGC

[a] Xhc-q2 primer set for TaqMan real-time polymerase chain reaction (PCR) assay designed from the genomic sequence targeted by the PCR primer pair XhcPP02 described by Kimbrel et al. (12).

[b] Lace primer set for loop-mediated isothermal amplification (LAMP) designed from the PCR product of the 9B primer set published by Meng et al. (16). F and B are outer primers, and Fip and Bip are inner primers; Loop F is a nested primer that in early cycles of DNA replication increases the number of starting points for FIP.

PMA treatment. Medium 523 broth cultures of *X. hortorum* pv. *carotae* strains M081^R or Car111 in log phase were pelleted (16,000 × *g* for 2 min), resuspended in sterilized distilled water, and adjusted to approximately 5×10^4 CFU/ml with the aid of a spectrophotometer. Cells in this suspension were subdivided into six replicate suspensions (1 ml each) of "live cells" (stored in microcentrifuge tubes at 4°C until needed) and six suspensions of "dead cells". To kill the cells, the bacterial suspensions in sealed microcentrifuge tubes were incubated in a hot-water bath (Isotemp Model 110; Thermo Fischer Scientific) at 52°C for 25 min. A 20 mM stock solution of PMA in 20% dimethyl sulfoxide was added to half of the live and dead cell suspensions (three each) to achieve a final concentration of 50 µM. Cell suspensions with PMA were then vortexed briefly and incubated in the dark for 5 min to allow the dye to intercalate with DNA. Microcentrifuge tubes holding the suspensions were then placed in chipped ice with the lids open and exposed for 5 min at a distance of 20 cm to light from a 650-W, sealed-beam halogen lamp (model FCX-Q650PAR36/6; General Electric Co.) set in a Type 5711 one-light Molefay fixture (Mole-Richardson Co.) to bind intercalated PMA to DNA and to inactivate unbound PMA. In samples prepared for real-time PCR assay but not for LAMP assay, PMA-treated cell suspensions were then subjected to the PMA protocol a second time (23). After the light exposure, bacterial cells in each microcentrifuge tube were pelleted, the supernatant aspirated, and the pellet resuspended in sterilized distilled water. Aliquots (10 µl) of live- and dead-cell suspensions were diluted serially (10-fold) into 0.015 M phosphate buffer and plated onto XCS agar to quantify viable cell concentration. The replicated 1-ml subsamples of nontreated, hot water, and PMA treatment combinations were subjected to DNA extraction as described above, and the real-time PCR and LAMP dilution endpoint assays were performed on DNA from each replicate suspension in triplicate.

The effect of PMA on a carrot seed wash was tested with a composite seed lot created by combining five lots of carrot seed produced in *X. hortorum* pv. *carotae*-inoculated research plots located near Madras, OR (provided by Dr. Bo Ming Wu). Six replicates of seed (10 g each) were each placed in a cheesecloth bag. Half the seed replicates were subjected to heat by submersion in 100-ml beakers of preheated (52°C), distilled water for 25 min, with the temperature maintained by holding the beakers in a heated water bath. After heat treatment, a seed wash of each replicate was prepared by soaking the seed overnight at 4°C in 100 ml of sterilized 0.85% NaCl. After soaking, each seed wash was amended with a drop of Tween-20, agitated for 5 min on a rotary shaker at 250 rpm, and poured through four layers of sterilized cheesecloth to remove the seed and debris from the wash, then poured through a 35-µm mesh screen to remove fine debris. The 100 ml wash was pelleted (10,400 × *g* for 10 min), the supernatant discarded, and the pellet resuspended in 10 ml of sterilized 0.85% NaCl (5,14). For each replicate, a 1-ml subsample of each hot-water-treated and nontreated seed wash was exposed to PMA as described above. Two 100-fold dilutions were made from each nontreated, PMA-treated, and hot-water-treated wash subsample by transferring 10 µl into 1 ml of sterilized phosphate buffer; 10 µl of each wash and of the two dilutions were each spread onto three plates of XCS agar in 9-cm-diameter petri dishes. Dilution plates were incubated for 5 to 7 days before bacterial populations were enumerated. Populations of *X. hortorum* pv. *carotae* were then assessed by counting colonies with morphology similar to reference colonies of strain M081^R or strain Car111 (light-yellow, round, mucoid colonies with smooth edges). Representatives of suspected *X. hortorum* pv. *carotae* colonies also were subcultured for verification with the LAMP or real-time PCR assays. After dilution plating, the 1-ml subsamples of the replicated treatment combinations (Fig. 1) were subjected to DNA extraction as described above. Detection of *X. hortorum* pv. *carotae* with real-time PCR and LAMP dilution endpoint assays was done on each subsample in duplicate.

Evaluation of molecular assays on 36 commercial carrot seed lots. In all, 36 carrot seed lots with different levels of *X. hortorum* pv. *carotae* contamination were obtained from commercial seed companies (Table 2); 11 of these seed lots had been treated with hot water or trisodium phosphate by the seed companies to reduce *X. hortorum* pv. *carotae* infestation. At both Mount Vernon and Corvallis, six 2.5- to 10-g replicates of each seed lot (depending on the amount of seed available per lot) were washed as described above; the volume of the wash was proportional to the size of the seed replicate. Subsamples of washes from the commercial seed aliquots were processed as described above for the composite seed lot, resulting in three replicates of each of the four seed wash treatment combinations (Fig. 1). The dilution plating was augmented by spreading 100 µl of each seed wash onto three plates of XCS agar in a 14-cm-diameter petri dish. At Corvallis, the InstaGene Matrix protocol was used exclusively for samples prepared for the LAMP assay. Three real-time PCR reactions per replicate were performed with undiluted DNA extracts. Similarly, for the LAMP assay, an initial reaction (three per replicate) was done with undiluted DNA extracts to determine the replicates that tested positive for *X. hortorum* pv. *carotae*. Subsequently, a 10-fold dilution series of extracted DNA was created for each replicate for LAMP dilution endpoint analysis; dilutions and corresponding LAMP reactions (three per replicate) were performed out to 1×10^{-7} for all subsamples that tested positive in the initial assay.

Linear regression analysis (Analyse-it Software, Ltd.) was used to compare molecular assays to colony counts obtained by dilution plating. Initially, to provide an expectation for correspondence between assays, the dilution plating results for commercial seed lots processed at Corvallis were regressed on results from the same

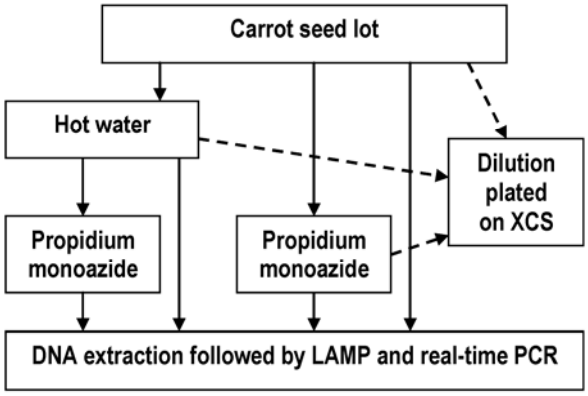

Fig. 1. Flow chart of the process used to evaluate two molecular assays versus a dilution plating assay to quantify the infestation level of *Xanthomonas hortorum* pv. *carotae* in carrot seed lots, as affected by treatment with hot water and the live-dead discriminating dye, propidium monoazide. PCR = polymerase chain reaction and LAMP = loop-mediated isothermal amplification.

Table 2. Countries of origin and levels of contamination by *Xanthomonas hortorum* pv. *carotae* for 36 commercial carrot seed lots tested with molecular assays for the bacterial blight pathogen developed in this study

	Level of infestation[a]		
Country of origin	**Low**	**Moderate**	**High**
United States	7 (6)	3 (3)	3
France	0	1	4
Chile	3 (1)	3	1
Argentina	0	3	0
New Zealand	1	0	0
South Africa	0	1 (1)	0
Unknown	2	3	1
Total	13	14	9

[a] Concentration of *X. hortorum* pv. *carotae*: Low = $\leq 1 \times 10^2$ CFU/g seed, Moderate = $>1 \times 10^2$ and $<1 \times 10^5$ CFU/g seed, and High = $>1 \times 10^5$ CFU/g seed. Number in parentheses represents seed lots treated by seed companies prior to being evaluated in this study. Seed treatments applied were hot water (for five seed lots), trisodium phosphate (three seed lots), or both (three seed lots).

seed lots processed at Mount Vernon. Because the seed lots were split and washed independently at the two locations, the unexplained variation observed in colony counts was considered a "between seed wash" baseline on which to compare the correspondence among dilution plating and molecular assays. In addition, at Corvallis, colony counts on dilution plates from PMA-treated seed wash subsamples were regressed on colony counts from the same but nontreated seed wash subsamples to determine the effect of PMA on live *X. hortorum* pv. *carotae*; because only a minor effect of PMA was observed, the unexplained variation in this relationship provided a baseline expectation for "within seed wash" variation. Following these analyses, the colony count data from the commercial seed lot washes were regressed on the respective estimates of *X. hortorum* pv. *carotae* CFU obtained with the real-time PCR and LAMP dilution endpoint assays.

Results

Primer selection and evaluation of specificity. For the nine *Xanthomonas* pathovars tested, only *X. hortorum* pv. *carotae* strains were amplified with the real-time PCR primers designed from a region of *X. hortorum* pv. *carotae* genomic DNA (12) (Table 1). In addition to *X. hortorum* pv. *carotae*, the LAMP primers designed from the 9B PCR product reported by Meng et al. (16) also amplified isolates of *X. campestris* pv. *coriandri*, as reported previously (12). No other strains were amplified using the PCR or LAMP primers designed for *X. hortorum* pv. *carotae* detection (see Kimbrel et al. [12] for a list of other bacteria evaluated).

Standard curves and sensitivity of the real-time PCR and LAMP assays. For the Qiagen blood and tissue kit, extraction of *X. hortorum* pv. *carotae* DNA from pure cultures of 1×10^6 to 1×10^9 CFU/ml yielded a linear relationship: $\log_{10}$ [dilution plate count (CFU/ml)] = $1.17 \times \log_{10}$ (DNA concentration) (coefficient of determination [R^2] = 0.97), where DNA concentration fg/µl) was measured with a Qubit fluorometer. Similarly, for the InstaGene matrix kit, extraction of *X. hortorum* pv. *carotae* DNA from pure cultures of 1×10^6 to 1×10^9 CFU/ml yielded a linear relationship: $\log_{10}$ [dilution plate count (CFU/ml)] = $1.06 \times \log_{10}$ (DNA concentration) (R^2 = 0.99), where DNA concentration (femtograms per microliter) was measured with a Nanodrop spectrophotometer. Also based on *X. hortorum* pv. *carotae* DNA extractions from pure cultures, the C_t values of the real-time PCR assay and the dilution endpoint of LAMP assays were linear with the $\log_{10}$ (CFU) (Fig. 2), which was estimated by conversion from $\log_{10}$ (DNA concentration) with the above equations. These standard curves showed that the detection limit of both molecular assays for *X. hortorum* pv. *carotae* DNA extracted from pure cultures was approximately 10^1 CFU/ml. Because the real-time PCR assay results became nonlinear when *X. hortorum* pv. *carotae* CFU were below this limit, a C_t value ≥ 35 (approximately 10^1 CFU/ml) was considered to be a negative sample in subsequent assays. For the LAMP assay, the method of DNA extraction (Qiagen DNeasy blood and tissue extraction kit versus the InstaGene matrix kit and spin column) did not influence DNA amplification. Consequently, the InstaGene matrix kit was used for DNA extraction from commercial seed lots for the LAMP assay.

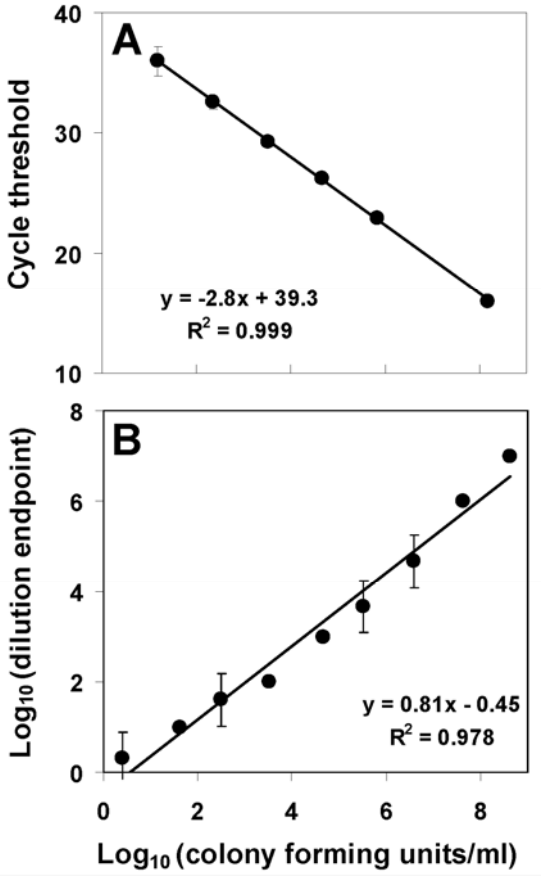

Fig. 2. Standard curves relating CFU of *Xanthomonas hortorum* pv. *carotae* from pure culture to quantitative statistics from two DNA detection assays: **A,** the cycle threshold value of a TaqMan real-time polymerase chain reaction (PCR) assay and **B,** the dilution endpoint of a loop-mediated isothermal amplification (LAMP) assay. Values of $\log_{10}$ (CFU/ml) of *X. hortorum* pv. *carotae* on the x-axis were estimated from $\log_{10}$ (DNA concentration [femtograms per microliter]) using conversion factors provided in the text. Error bars represent ± one standard deviation of the mean.

PMA treatment and DNA extraction. For the cell suspension prepared from a pure culture, *X. hortorum* pv. *carotae* was recovered on XCS agar medium at populations averaging 5.02 ± 0.27 (mean ± standard deviation) and $4.68 \pm 0.07 \times \log_{10}$ (CFU/ml) for nontreated and PMA-treated samples, respectively (Fig. 3A). Similarly, for the composite seed lot infested with *X. hortorum* pv. *carotae*, the pathogen was recovered at populations averaging 5.10 ± 0.13 and $4.97 \pm 0.12 \times \log_{10}$ (CFU/ml) for nontreated and PMA-treated samples, respectively (Fig. 3D). Dilution plating confirmed that hot-water-treated *X. hortorum* pv. *carotae* cell suspensions from pure cultures or from the composite seed lot were nonviable because no colonies were recovered on XCS agar medium.

Positive real-time PCR and LAMP assay results were observed for DNA extracted from all viable suspensions of PMA-treated and nontreated *X. hortorum* pv. *carotae* cells, and from all suspensions of hot-water-treated (nonviable) cells that were not treated with PMA (Fig. 3B, C, E, and F). Moreover, for samples not treated with hot water, the molecular assays showed only small differences (less than one-third log unit) in estimated number of *X. hortorum* pv. *carotae* CFU compared with the dilution plating results (Fig. 3). For both the pure culture suspension and the pathogen-infested seed lot, negative real-time PCR and LAMP assays were obtained for DNA extracts from *X. hortorum* pv. *carotae* cells treated with hot water and PMA prior to DNA extraction (Fig. 3B, C, E, and F).

Evaluation of molecular assays on 36 commercial carrot seed lots. Based on dilution plating, commercial carrot seed lots obtained from seed companies had levels of *X. hortorum* pv. *carotae* infestation that ranged from not detectable to 2×10^7 CFU/ml seed wash. Also, by visual inspection, the relative cleanliness of the seed lots varied widely, ranging from clean (tan-colored seed without debris) to 1 to 2% debris and dark discoloration of the seed. Eleven of the seed lots had been treated previously with hot water, trisodium phosphate, or both, which resulted in a cleaner appearance and a lower level of *X. hortorum* pv. *carotae* detected compared with nontreated seed (company-treated seed averaged 6 CFU/g seed, whereas nontreated seed averaged 600 CFU/g).

Compared with dilution plating, the LAMP and real-time PCR assays applied to nontreated seed showed a greater number of positive seed lots and detected a greater mean estimate of *X. hortorum* pv. *carotae* CFU per gram of seed, which was likely due, at least in part, to detection of DNA from dead pathogen cells in seed lots that had been treated by the companies (Table 3). Similarly, after hot-water treatment, all seed lots tested negative for *X. hortorum*

pv. *carotae* by dilution plating on XCS agar but approximately three-fourths of the seed lots remained positive for *X. hortorum* pv. *carotae* with the real-time PCR and LAMP assays. For all three assays, treatment with PMA but not hot water resulted in approximately two-thirds of the seed lots testing positive for *X. hortorum* pv. *carotae*, with each method providing a similar estimate of the mean infestation level (note: for dilution plating and real-time PCR assays, this result required an arbitrary definition of a positive and negative assay, as described in the footnotes to Table 3). For hot-water treatment of seed followed by PMA treatment of the seed wash, none of the LAMP assays was positive, and 5 of 32 seed lots tested positive by real-time PCR assay with minimal *X. hortorum* pv. *carotae* infestations for those five positive lots (i.e., <2.3 × $\log_{10}$ CFU/g seed) (Table 3).

After dividing the commercial seed lots among the Corvallis and Mount Vernon labs, regression of the logarithm of *X. hortorum* pv. *carotae* CFU obtained by dilution plating showed a significant ($P < 0.01$) linear relationship among the seed washes processed at each location (Fig. 4A); however, the R^2 revealed a relatively large amount of unexplained variation ($R^2 = 0.75$; Fig. 4A). The regression of dilution plating results from the same seed washes before and after treatment with PMA also resulted in a significant ($P < 0.01$) linear relationship (Fig. 4B). Dilution plate counts of *X. hortorum* pv. *carotae* CFU were reduced by approximately 5% after PMA treatment compared with prior to PMA treatment but the amount of variation explained by the regression relationship was high ($R^2 = 0.96$).

For nontreated seed, regression of the logarithm of *X. hortorum* pv. *carotae* CFU obtained by dilution plating versus the logarithm of the estimate obtained by a molecular assay resulted in significant ($P < 0.01$) linear relationships for both the real-time PCR and LAMP dilution endpoint assays ($R^2 = 0.56$ and 0.85, respectively; Fig. 5A and B). The seed lots pretreated by the seed companies to reduce *X. hortorum* pv. *carotae* infestations were excluded from

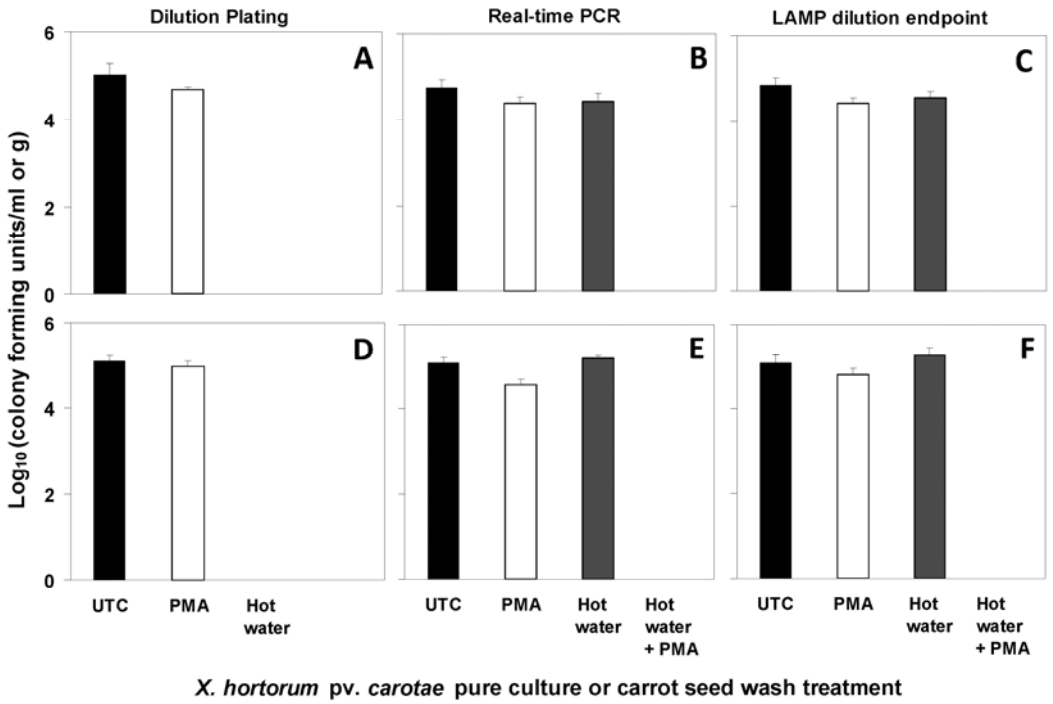

Fig. 3. Estimates of *Xanthomonas hortorum* pv. *carotae* CFU detected in **A–C,** suspensions prepared from pure culture (per milliliter) or **D–F,** a wash of pathogen-infested carrot seed (per gram) based on **A** and **D,** colony counts after dilution plating onto the semiselective agar medium, XCS; **B** and **E,** the cycle threshold value of a TaqMan real-time polymerase chain reaction (PCR) assay; or **C** and **F,** the dilution endpoint of a loop-mediated isothermal amplification (LAMP) assay. Treatments applied to the *X. hortorum* pv. *carotae* suspensions were untreated (UTC); the live-dead discriminating dye, propidium monoazide (PMA); hot water (52°C for 25 min); and hot water followed by PMA. Means shown are the average of three replications with two subsamples per replication; error bars represent one standard deviation among the replications.

Table 3. Summary statistics for three methods of detection of *Xanthomonas hortorum* pv. *carotae* in seed washes from 36 commercial carrot seed lots subjected to treatment with hot water, propidium monoazide (PMA), or hot water followed by PMA

	Method of detection of *X. hortorum* pv. *carotae*[a]					
	Dilution plating assay		LAMP assay		Real-time PCR assay	
Treatment[b]	Number of positive seed lots[c]	Mean $\log_{10}$ (CFU/g of seed)	Number of positive seed lots[d]	Mean $\log_{10}$ (dilution endpoint)	Number of positive seed lots[e]	Mean $\log_{10}$ (CFU/g of seed)
Nontreated	25 of 36	2.92 (1.87)	31 of 36	3.46 (1.61)	34 of 36	4.58 (1.86)
PMA	23 of 36	3.15 (1.97)	24 of 36	3.14 (1.67)	23 of 36	3.21 (1.51)
Hot water	0 of 36	…	30 of 36	3.50 (1.56)	26 of 34[f]	4.31 (1.72)
Hot water, then PMA	…	…	0 of 36	…	5 of 34	2.26 (0.34)

[a] Methods were a dilution plating assay onto the semiselective agar medium XCS, a loop-mediated isothermal amplification assay (LAMP), and a TaqMan real-time polymerase chain reaction (PCR) assay. Mean $\log_{10}$ given if positive. Numbers in parentheses indicate standard deviation of the mean.
[b] Treatment applied to seed. Seed companies that supplied the seed lots had treated 11 of the 36 seed lots with hot water, trisodium phosphate, or both (see Table 1).
[c] Positive seed lot defined as $\log_{10}$ (CFU/g carrot seed) > 0.5.
[d] Positive seed lot defined as the presence of turbidity in the LAMP reaction tube (21).
[e] Positive seed lot defined as a cycle threshold value < 35.0.
[f] Total number of seed lots tested was reduced to 34 owing to insufficient seed available for hot water treatment of two seed lots.

these analyses. PMA treatment of the seed samples improved the regression of the logarithm of *X. hortorum* pv. *carotae* CFU detected by dilution plating versus the logarithm of the estimated CFU detected by real-time PCR assay (R^2 = 0.68; Fig. 5C). However, the amount of explained variation obtained by regressing the dilution plating results on the LAMP dilution endpoint assay results declined slightly (R^2 = 0.79) for seed samples treated with PMA compared with the same regression with the nontreated seed samples (Fig. 5D).

Discussion

For producers of carrot seed in the Pacific Northwest United States, *X. hortorum* pv. *carotae* is a perplexing problem given that the severity of bacterial blight symptoms observed on leaves and umbels is typically negligible but the harvested seed frequently is infested with high populations of the pathogen. This is due to a polycyclic, epiphytic phase of the bacterium that builds logistically as the season progresses (5). Methods to suppress this epiphytic phase include sanitation (clean planting material and prebloom sprays of copper-based bactericides) and the use of drip irrigation to avoid wetting the phyllosphere during bloom and seed maturation (5). These methods, however, are only partially effective and, consequently, pathogen testing and hot-water sanitation have become routine industry practices for providing clean seed for carrot root crop production (5–7). Currently, the standard method for *X. hortorum* pv. *carotae* detection in carrot seed involves dilution plating the seed wash onto a semiselective agar medium such as XCS (1,6,10,13). Although the accuracy of dilution plating is considered sufficient, a minimum of 5 days is required to visualize *X. hortorum* pv. *carotae* colonies on the selective medium (1,5,6,10,13,31). Moreover, positive identification of *X. hortorum* pv. *carotae* on the plates can require further verification due to the presence of saprophytic bacteria on seed that have colony morphologies similar to that of the pathogen (1,6,16). Thus, dilution plating of seed wash is the standard for quantitative detection of *X. hortorum* pv. *carotae* but requires a significant period of time (1,6,16). In this study, one of the goals was to reduce the time required to estimate the number of viable *X. hortorum* pv. *carotae* cells in carrot seed lots with a precision that was similar to the plating assay.

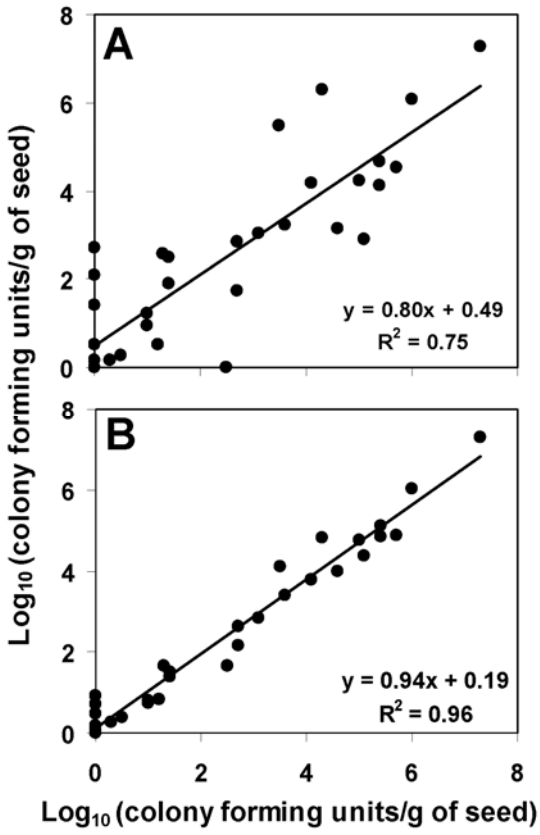

Fig. 4. Regression of *Xanthomonas hortorum* pv. *carotae* CFU cultured from washes of 36 commercial carrot seed lots. **A,** Results from dividing the seed lots, and then washing and assaying the seed at different laboratory facilities: Corvallis, OR (X-axis) and Mount Vernon, WA (Y-axis). **B,** Results from seed washes prepared and assayed in Corvallis before (X-axis) and after (Y-axis) treatment of the seed wash with the live-dead discriminating dye propidium monoazide.

Dilution plating onto XCS agar demonstrated that PMA treatment at 50 μM *X. hortorum* pv. *carotae* suspensions in water or 0.85% NaCl was not toxic to the pathogen, because the populations were not affected significantly by the treatment. Additionally, the colony counts from commercial seed washes were only 5% less for PMA-treated seed washes compared with nontreated washes. These results agree with those of Nocker et al. (19,20), who documented no toxicity of PMA to a range of bacterial species. It was also important to verify that the use of PMA on nonviable *X. hortorum* pv. *carotae* suspensions from pure cultures and hot-water-treated carrot seed resulted in no amplification of DNA with the LAMP assay or with real-time PCR assay, using $C_t \leq 35$ to define a positive reaction. Following the recommendation of Pan and Breidt (23), carrot seed washes subjected to real-time PCR assays were treated twice with PMA, whereas those subjected to the LAMP assay were only treated once. Paradoxically, it was the real-time PCR assay that showed a greater number of false-positive samples after hot water then PMA treatments compared with the LAMP assay. This result perhaps reflects differences in assay performance or sensitivity at low levels of target DNA but also shows that the first treatment of the seed wash with PMA rendered nonreactive most of the DNA that was not associated with viable *X. hortorum* pv. *carotae* cells. Optimization of the PMA concentration and perhaps other components of the assay protocols (e.g., if no hot-water treatment is involved, seed lots could be prerinsed prior to soaking) should be investigated further if a molecular assay is adopted for routine screening of seed lots.

In aggregate, the regression relationships in this study revealed close achievement of the goal of a molecular assay with a level of precision similar to the dilution plating assay. Regressions of colony counts detected with the dilution plating (Fig. 4) assay characterized among and within seed wash variation. Ideally, because dilution plating and molecular assays were performed within each seed wash, *X. hortorum* pv. *carotae* colony counts regressed on the estimates of CFU per gram of seed based on molecular assays after treatment with PMA (Fig. 5C and D) should show approximately the same amount of unexplained variation as dilution plate assays of the same seed wash before and after PMA treatment (Fig. 4B). In reality, the precision achieved was more like that observed for the "among seed wash" variation depicted in Figure 4A. The implication of this greater variability is that additional biological replications of molecular assays will be needed to achieve the level of precision of the dilution plating assay. In the regression analyses, the LAMP dilution endpoint assay gave slightly better R^2 values than the real-time PCR assay, especially for the seed washes not treated with PMA. It has been noted that LAMP is less sensitive than PCR to inhibitors in environmental samples (11,17), which may explain part of the difference observed between the two molecular methods. Results with the real-time PCR assay were improved when the seed washes were treated with PMA; this additional treatment (including a centrifugation step) likely removed some of the variability associated with debris and nontarget microorganisms in the seed washes and, potentially, some PCR inhibitors (15,26) also were removed. In contrast, for the LAMP dilution endpoint assay, the seed wash was diluted at each step but evidence for an improvement of assay precision at greater levels of dilution was not apparent.

A presence-or-absence dilution series assay (i.e., the LAMP assay) is a fundamentally different approach than attempting to achieve a precise C_t for a given concentration of target DNA (the real-time PCR assay) in a variable environmental background such

as carrot seed washes. As a case in point, in addition to LAMP dilution endpoint, we also evaluated a DNA dye-based assimilation probe (no turbidity), real-time LAMP assay (OptiGene Limited) (3,9) on the nontreated and PMA-treated DNA extracted from the commercial carrot seed lots (*data not shown*). The real-time LAMP assay utilizes a time threshold concept similar to the C_t of real-time PCR assays (9,11,17,34). The regression relationships obtained for the real-time LAMP assay were of poor quality (R^2 = 0.2 to 0.5), which was related to inconsistency in the time threshold values from both the samples and the standard curve (9,11). A SYBR Green real-time PCR assay was a fourth molecular assay that we evaluated on DNA extracted from the commercial seed lots (*data not shown*). This dye-based system applied to the seed samples also demonstrated a high degree of unexplained variation (R^2 = 0.4 to 0.5), particularly at low to moderate levels of target DNA in the seed washes; for the SYBR Green assay, C_t values rarely exceeded 30 and became nonlinear at 0 to 1,000 CFU/g of seed. That both the TaqMan real-time PCR assay and the dilution endpoint LAMP assay were reasonably precise (given two very different approaches to DNA quantification), suggests that a hybrid approach of a seed wash dilution followed by a C_t estimation could potentially enhance precision. For example, from a carrot seed wash, one 1,000-fold or two 100-fold dilutions could be made, and then a real-time PCR assay or perhaps a real-time LAMP assay used to estimate CFU over the smaller potential range of target DNA within each level of dilution.

A secondary issue, and perhaps less important in commercial seed production, was the issue of selecting criteria for calling a sample negative or positive, given that the data were compared among assays with different detection limits. In this regard, the TaqMan real-time PCR assay was most problematic because it became nonlinear with dilution plate colony counts below 10 CFU/ml ($35 < C_t < 40$). Consequently, a C_t of 35 (approximately 10 CFU/ml) was chosen as the cutoff for a negative sample. Similarly, for dilution plating, especially using large petri plates onto which 100 µl of seed wash was spread, several seed lots had very low mean numbers of *X. hortorum* pv. *carotae* recovered (3 to 5 CFU/g, which was below the detection limit of both the real-time PCR and LAMP assays). Consequently, we selected $\log_{10}$ (CFU/g) = 0.5 as the cut-off between a positive and a negative sample.

In conclusion, this study demonstrated that the DNA-intercalating dye, PMA, can be used to eliminate DNA from nonviable *X. hortorum* pv. *carotae* cells from detection by real-time PCR and LAMP assays. The study also demonstrated that both real-time PCR and LAMP assays can detect *X. hortorum* pv. *carotae* consistently and quantitatively from carrot seed washes. The primary advantages of a PMA-real-time PCR assay are a directly quantifiable, highly specific result from a single sample in a short period of time (6 to 8 h). With a PMA-LAMP assay, the advantages are a consistent and specific presence-or-absence test for viable *X. hortorum* pv. *carotae* coupled with low cost and an even more rapid assay time. By increasing the timeliness and efficiency of pathogen testing, real-time PCR and LAMP assays have the potential to contribute to bacterial blight prevention in regions of carrot seed and root crop production.

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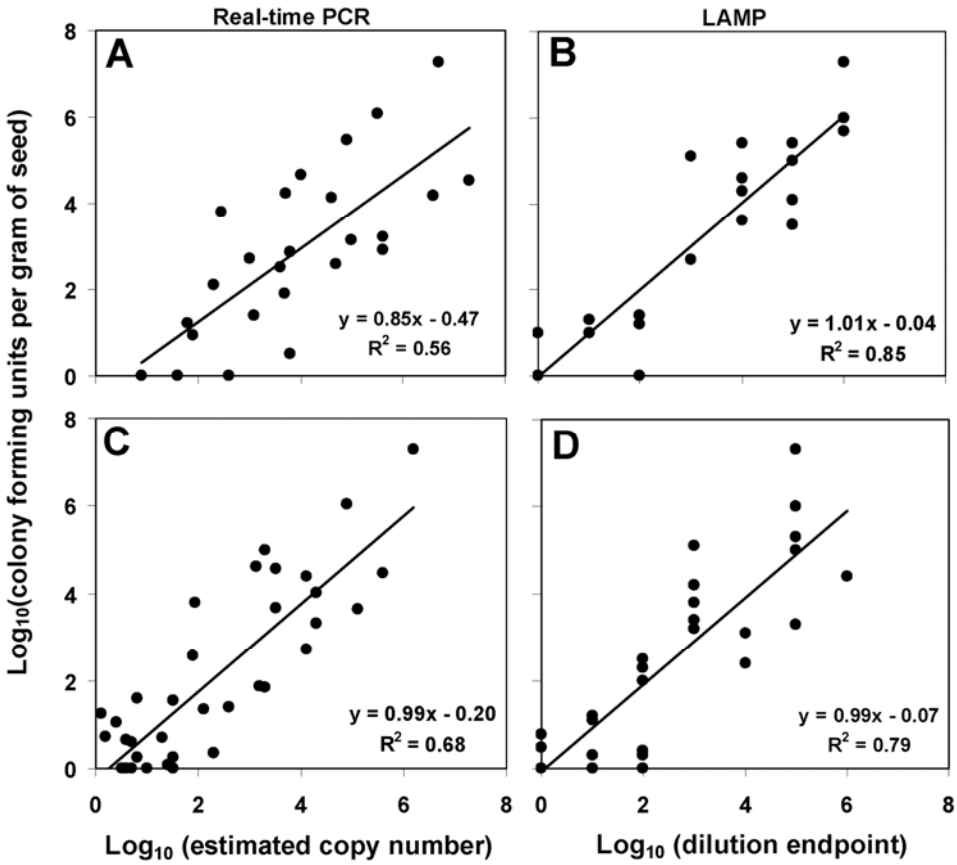

Fig. 5. Regression of *Xanthomonas hortorum* pv. *carotae* CFU cultured from washes of commercial carrot seed lots on a molecular assay performed on DNA extracted from the same seed washes. **A** and **C,** Estimated CFU based on a TaqMan real-time polymerase chain reaction (PCR) assay; and **B** and **D,** dilution endpoint of a loop-mediated isothermal amplification (LAMP) assay. **A** and **B,** Nontreated carrot seed lots (n = 25) and **C** and **D,** carrot seed lots treated with the live-dead discriminating dye propidium monoazide (n = 36). Number of nontreated seed lots was smaller owing to treatment (hot water, trisodium phosphate, or both) by seed companies prior to receipt of the seed samples for this study.

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