Evaluation of Loop-Mediated Isothermal Amplification for Rapid Detection of *Erwinia amylovora* on Pear and Apple Fruit Flowers

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Abstract

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Fire blight of pear and apple is frequently an inoculum-limited disease but weather-based forecasting models commonly assume that the pathogen is omnipresent. To improve fire blight risk assessment during flowering, we developed a rapid pathogen detection protocol that uses loop-mediated isothermal amplification (LAMP) to detect DNA of epiphytic *Erwinia amylovora* on samples of pear and apple flowers. LAMP detected a single flower colonized epiphytically by *E. amylovora* in a sample of 100 flower clusters (approximately 600 flowers). Samples of 100 flower clusters from orchards (approximately one sample per hectare) were washed and subjected to LAMP, which was completed in 2 h. In three experimental orchards inoculated with *E. amylovora*, positive LAMP reactions were attained from nine of nine 100-flower cluster samples; pathogen populations in the floral washes averaged 5.2×10^3 CFU per flower as determined by dilution plating. Samples of pear and apple flowers obtained from 60 commercial or-

chards located in Oregon, Washington, California, and Utah resulted in detection of *E. amylovora* by LAMP assay from 34 sites, 20 of which developed fire blight. Of samples at early bloom, 10% were positive for epiphytic *E. amylovora* compared with 28% at petal fall; pathogen density in washes of positive samples averaged 3.2×10^2 CFU per flower. In another 26 orchards, all floral washes were negative for *E. amylovora* by LAMP and by dilution plating; a light severity of fire blight was observed in 8 of these orchards. Overall, positive detection of epiphytic *E. amylovora* in commercial orchards by LAMP-based scouting generally occurred at later stages of bloom after heat (risk) units had begun to accumulate, an indication that weather-based forecasting models may be an adequate measure of fire blight risk for many orchardists. Nonetheless, several orchardists communicated that information from the LAMP-based rapid detection protocol resulted in modification of their fire blight management practices.

With the development of molecular tools for pathogen detection, there has been increased interest in using these tools in tactical disease management (1,14,17,19,20,35). An important constraint to this purpose, however, is that many detection technologies require special equipment or processing with associated expense and delay. For example, with fire blight of pear and apple, protocols have been developed previously for detection of *Erwinia amylovora* through use of culture media (9,15,23,34), enzyme-linked immunosorbent assay (7), and DNA amplification from floral washes via polymerase chain reaction (PCR; 2,21,29) but the methods are not used widely to determine the need for protective sprays because of issues with processing expense, assay sensitivity, or the time required to get an answer. The interest in obtaining knowledge of the presence or absence of epiphytic populations of *E. amylovora* on flowers relates to the significance of floral infection as a primary focal point in fire blight suppression (11,31), and to the recognized sporadic nature of this highly destructive disease (3,34). Sporadic outbreaks of fire blight are largely attributable to variability in epiphytic populations of the pathogen on flowers. In the Pacific Northwest region of the United States, the pathogen frequently is not present or is below damaging levels in orchards, even after weather-based forecasting models signal a risk of infection and the need for protective antibiotic treatments (5,30).

The objectives of this research were to develop a rapid detection technology for *E. amylovora* and evaluate its potential for use in tactical disease management. The method of DNA detection we evaluated is termed loop-mediated isothermal amplification (LAMP; 27). Similar to PCR, this technique uses primers to detect

and amplify sequences of target DNA. Unlike PCR, a LAMP reaction does not require a thermocycler to carry the DNA amplification through specific temperature phases. Instead, the reaction is done isothermally (typically at 65°C) in a heat block or water bath. LAMP has been shown to detect as few as 10 cells of a target organism (femtogram amounts of DNA) in a 1-h reaction (24). LAMP protocols have been developed previously to detect plant pathogens in environmental samples (13,16,25,26,36). Recently, LAMP has been adapted for use with lateral-flow devices (36), which offers the potential for on-site use by nonspecialists (4,24,35).

Materials and Methods

Bacterial strains. LAMP protocols for *E. amylovora* were developed using strain Ea153N, which is a spontaneous, nalidixic-acid-resistant (100 µg/ml) mutant of a strain originally isolated from a canker on apple collected in Milton-Freewater, OR. Ea153N has been used as a representative isolate in numerous field studies (10,12,32). Other bacterial strains used in this study are listed in Table 1; these strains, from a culture collection maintained by V. O. Stockwell and J. E. Loper (United States Department of Agriculture–Agricultural Research Service Horticultural Laboratory, Corvallis, OR), were used to evaluate the specificity of developed LAMP primers to *E. amylovora*.

DNA extraction. Bacterial cells in suspension (1 ml) were pelleted by centrifugation (14,000 rpm for 2 min) and resuspended in 200 µl of InstaGene matrix (Bio-Rad Laboratories, Inc., Hercules, CA). The cell mixture was vortexed for 5 s and incubated at 56°C for 20 min followed by boiling for 8 min. After boiling, the mixture was centrifuged for 2 min at 10,000 rpm, and the supernatant was transferred to a mini-elute column (Epoch Biolabs, Inc., Sugarland, TX) and centrifuged for 1 min at 14,000 rpm.

LAMP. The LAMP protocol followed methods of Notomi et al. (27), with several modifications. Individual reactions were done in a 50-µl volume consisting of a reagent mix, DNA, and polymerase. The reagent mix contained (with final concentrations) internal primers termed FIP and BIP (24,27) (2.4 µM), outer primers termed F and B (0.2 µM), loop primer (0.4 µM when used), dNTPs

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(1.0 mM), betaine (0.8 M), bovine serum albumin (0.4 mg/ml), ThermoPol buffer (1x; New England BioLabs, Beverly, MA), and MgSO₄ (2 mM); this mix was allocated to each assay tube before the addition of double-stranded template DNA. Cell extracts (5 µl) were used as template DNA; sterile water was used in the negative controls. Finally, 16 units (2 µl) of *Bst* DNA polymerase (New England BioLabs, Ipswich, MA) were added to the reaction mix, followed by incubation at 65°C in a water bath or heat block. After 1 h, reactions were assessed for DNA amplification by visual observation of turbidity in the assay tube; the turbidity is caused by a precipitate of magnesium pyrophosphate and is indicative of a positive LAMP assay (24,27).

LAMP primer design. The DNA sequence of *E. amylovora* plasmid pEA29 was obtained from the National Center for Biotechnology Information (accession AF264948; 22). From this sequence, numerous sets of primers meeting the specifications for LAMP amplification were designed with the PrimerExplorer V4 software program (Fujitsu Ltd., Tokyo; <http://primerexplorer.jp>). Additional primers targeted to the chromosome of *E. amylovora* also were designed and evaluated but are not reported on here.

After initial testing, a LAMP primer set targeted to pEA29 of Ea153N was selected for further evaluation. Amplified products from positive LAMP assays were purified using a PCR product clean-up kit (ExoSAP-IT; USB Corp., Cleveland) and sequenced by the Oregon State University Center for Genome Research and Biocomputing. Sequenced products were compared with the pEA29 sequence used for LAMP primer design. LAMP products also were subjected to electrophoresis on agarose gels to establish a banding pattern to be used to confirm positive reactions in later experiments (27). The products were prepared for electrophoresis by double digestion with the restriction enzymes *Mlu*I and *Hae*III (New England Biolabs, Ipswich, MA); digested LAMP products were run in 0.7 or 2.0% agarose at 70 V for 1 h and visualized on an ultraviolet gel imager (UVP Transilluminator, Cambridge).

Primer sensitivity and specificity. Sensitivity of the pEA29 LAMP primer set was assessed by observing amplification of DNA extracted from a serial dilution of cultured Ea153N, and also by serial dilution of extracted DNA. Dilution endpoint turbidity was evaluated with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE) at an optical density of 400 nm (24,27), and by visual inspection. Specificity of the pEA29 LAMP primer set was tested against DNA from various species of plant-associated enteric bacteria and other species commonly associated with pome fruit flowers (28,33), including additional isolates of *E. amylovora* (Table 1). Cell suspensions of these species were prepared at 5×10^5 CFU/ml and boiled for 10 min; 5 µl of a boiled suspension was used as a template for LAMP as described above.

Effect of flowers on LAMP detection of *E. amylovora*. Flowers of pear 'Bartlett' or apple 'Gala' or 'Red Delicious' were col-

lected in April 2008 and 2009 from experimental orchards located at the Oregon State University, Department of Botany and Plant Pathology Field Laboratory near Corvallis. Distilled water (3 liters) in plastic pails was amended with flowers at densities of 0, 10, 100, or 1,000 per pail. Freeze-dried Ea153N (with known titer) was added to the pails and thoroughly mixed with the water and flowers to achieve final concentrations of approximately 0, 500, or 5,000 CFU/ml at each flower density. Subsamples (1 ml) of each suspension was diluted serially onto CCT medium (9) amended with nalidixic acid at 75 µg/ml to enumerate Ea153N, and onto *Pseudomonas* agar F (PAF; Difco Laboratories, Detroit) amended with cycloheximide at 50 µg/ml to enumerate total bacterial populations. Samples were prepared for LAMP by first filtering 1 liter of each suspension through four layers of cheesecloth and then through a 35-µm microsiege (Biodesign Inc., Carmel, NY). The filtered suspension (15 to 30 ml) was concentrated by embedding on a 0.2-µm membrane (Supor 200; Pall Life Sciences, Port Washington, NY). Cells captured on the membrane were resuspended in 1 ml of sterile water, which was subjected to DNA extraction and a LAMP assay as described above. The experiment was replicated five times with both pear and apple flowers.

Single infested flower experiment. Stigmas on individual, newly opened, detached pear (Bartlett) or apple (Gala) flowers were inoculated with a suspension of Ea153N prepared at 5×10^5 CFU/ml. Inoculated flowers were held in 2-ml plastic tubes with the proximal end of the pedicel (1.5 to 2 cm in length) submerged in water and incubated for 3 to 4 days at 16°C inside of a plastic crispier box lined on the bottom with moistened paper towel. After incubation, stigmas of inoculated flowers were removed and placed in 1 ml of sterile water; a 10-µl subsample was dilution plated onto CCT medium to estimate the pathogen population size. The wash and stigmas were then added to either 1.5 liter (2008) or 300 ml (2009) of tap water or to 100 flower clusters (field-collected, 6 flowers per cluster) in 1.5 liter (2008) or 300 ml (2009) of tap water, which were held in 33-by-45-cm, resealable plastic bags (Minigrip, Kennesaw, GA). Water-alone and noninoculated 100-flower cluster samples in water were included as controls. Bags with flowers were vigorously hand massaged for 15 s, followed by sonication (Model 2200; Branson, Danbury, CT) for 2 min. As described above, the wash suspensions were prefiltered, embedded on a 0.2-µm membrane, resuspended in 1 ml of water, and subjected to DNA extraction and a LAMP assay.

Experimental orchard sampling. Pear and apple flowers from research orchards (0.25 to 0.5 ha) located at the Botany and Plant Pathology Field Laboratory were sampled and subjected to LAMP assay. Some orchards were being used for fire blight control experiments and had been inoculated with Ea153N at full bloom by misting each tree with 2 liters of an aqueous suspension prepared at 5×10^5 CFU/ml. Flowers for the LAMP assay were sampled 4 (2008

Table 1. Specificity evaluation of primers designed for detection of *Erwinia amylovora* by loop-mediated isothermal DNA amplification (LAMP) with plant-associated enteric bacteria and other species commonly associated with pome fruit flowers

Strain identity ^a	No. of strains with positive LAMP assay of total strains tested	
	LAMP pEA29 primer set	LAMP pEA29 primer set with loop primer
<i>Bacillus cereus</i>	0 of 2	1 ^b of 2
<i>B. subtilis</i>	0 of 1	0 of 1
<i>Dickeya</i> spp.	0 of 1	0 of 1
<i>Erwinia amylovora</i>	8 of 8	8 of 8
<i>E. amylovora</i> (lacking plasmid pEA29)	0 of 2	1 ^b of 2
<i>E. persicina</i>	0 of 2	0 of 2
<i>E. tasmaniensis</i>	0 of 2	0 of 2
<i>Methylobacterium</i> spp.	0 of 1	0 of 1
<i>Pantoea agglomerans</i>	0 of 3	2 ^b of 3
<i>Pectobacterium atrosepticum</i>	0 of 3	0 of 3
<i>Pseudomonas fluorescens</i>	0 of 1	0 of 1
<i>P. putida</i>	0 of 1	0 of 1
<i>P. syringae</i>	0 of 3	0 of 3

^a Based on sequencing of the 16S-23S rRNA intergenic spacer region.

^b Possible mispriming.

and 2009) and 9 (2008 only) days after inoculation. Noninoculated orchards were sampled at the same times. In each orchard, three 100-flower cluster samples were collected in 33-by-45-cm resealable plastic bags. Flower clusters were collected haphazardly along W-shaped transects through the orchards. Several clusters were sampled from most trees in each block. In the laboratory, the 100-flower cluster samples were washed and processed for dilution plating and LAMP assay as described in previous sections; the floral wash volume was 1.5 liter of water in 2008 and 300 ml in 2009.

Commercial orchard survey. From 2008 to 2010, flowers from 54 commercial pear and apple orchards (most of which were approximately 5 ha in size and located in major fruit-production districts of Oregon, Washington, and California) were sampled and subjected to LAMP and dilution-plating assays. Orchards selected for sampling were identified with the help of cooperators in each fruit-production district. The sampling methodology was similar to that used in experimental orchard blocks in Corvallis, except that the cooperators were instructed to take five 100-flower cluster samples in each orchard (one per hectare per sampling date) and to sample each orchard three times: mid-bloom, full bloom, and petal fall. However, because of time constraints for some cooperators, approximately 25% of orchards were sampled less thoroughly; however, at a minimum, orchards in this study were sampled twice with at least three 100-cluster samples per orchard on each sampling date. A W-shaped sampling transect was used to collect the samples; generally, one cluster was collected every few trees. Flower samples were shipped overnight to Corvallis for processing in our laboratory. Flowers were washed in 1.5 liters of water in 2008 but wash volumes were reduced to 300 ml in 2009 and 2010 based on inconsistent results between the LAMP assay and dilution-plate methods in 2008. Inconsistency among methods in 2008 was rectified by a fourfold SpeedVac (model DNA 120; Thermo Savant, Holbrook, NY) concentration of extracted DNA prior to the LAMP assay. In 2009, additional flower samples were collected from six apple orchards in Utah. Sampling in Utah differed from the other locations in that only one 100-flower cluster sample was collected per orchard per sample date but sampling was done daily, resulting in 10 to 12 samples per orchard.

Data on the development of fire blight in sampled orchards were collected by cooperators in late June to early July. Orchards were inspected and scored for fire blight severity: none, light (≤ 1 infection [strike] per tree), medium (2 to 5 strikes per tree), and heavy (≥ 6 strikes per tree).

Data analysis. Data from experiments to evaluate LAMP sensitivity were plotted as the concentration of template from Ea153N (CFU or concentration of extracted DNA) and turbidity as measured by the NanoDrop spectrophotometer (optical density at 400 nm [OD₄₀₀]). Because LAMP assay is a presence or absence test, assay results from laboratory experiments and the experimental orchards yielded qualitative results with no variation among replications; thus, data were simply tabulated. Estimates of the population size of *E. amylovora* recovered on dilution plates were transformed log₁₀ (y) (8) and reported as mean log (CFU) per milliliter or per flower as dictated by the experiment; arithmetic means presented in the text were backtransformed from mean log (CFU) per milliliter or per flower.

Results of LAMP assays and dilution plating performed on 100-flower cluster samples from commercial orchards were summa-

rized by production district and by bloom stage at the time of sampling. The value of the CougarBlight (30) risk assessment model also was calculated for each sampling date. For this purpose, the temperature data for each fruit district were obtained from records of local cooperative weather networks, which are maintained in databases by the Oregon State University Integrated Plant Protection Center. Dates with a negative detection or with one or more positive detections of *E. amylovora* by LAMP were plotted along composite, seasonal summaries of the CougarBlight risk index. Composite summaries were accomplished by plotting CougarBlight values for production areas in California, Oregon, and Washington for the time period when they were being sampled; risks values were averaged during periods when more than one district was sampled.

Results

Evaluation of sensitivity and specificity. The LAMP primer set (i.e., primers Fip, Bip, F, and B but not the loop primer) targeted to plasmid pEA29 of *E. amylovora* (Table 2) provided positive reactions for strain Ea153N and other strains of *E. amylovora* with pEA29 but did not react with two pathogen strains that lacked the plasmid (Table 1). The sequenced LAMP products from Ea153N showed 100% homology to the sequence for which the primers were designed. A serial dilution of cultured cells of Ea153N resulted in positive LAMP reactions from 9.9×10^9 to a statistically significant ($P \leq 0.05$) endpoint of 200 CFU/ml, as determined by the Nanodrop spectrophotometer (Fig. 1A). At cell densities of 2 and 20 CFU/ml, however, results of the LAMP assays were concluded to be negative (absorbance values statistically similar to water controls: 0.003 to 0.017, respectively, at OD₄₀₀). Overall, macroscopic detection of turbidity produced by a positive LAMP assay also required a cell suspension of ≥ 200 CFU/ml. The dilution series of DNA from *E. amylovora* resulted in positive LAMP assays at concentrations ranging from 114 $\mu\text{g}/\mu\text{l}$ to an endpoint of 500 $\text{fg}/\mu\text{l}$ (Fig. 1B). Macroscopic visualization of turbidity required ≥ 5 $\mu\text{g}/\mu\text{l}$ of DNA. Water controls were negative for turbidity by spectrophotometer or amplifiable products when run on an agarose gel (data not shown).

None of the other bacterial strains tested reacted positively with the pEA29-targeted primer set that included Fip, Bip, F, and B (Table 1). The addition of a loop primer (Table 2), however, resulted in positive amplification with one of the two strains of *E. amylovora* lacking pEA29, one of the two strains of *Bacillus cereus*, and two of the three strains of *Pantoea agglomerans* (Table 1). As a result, use of the loop primer was discontinued.

Effect of flowers on detection of the pathogen with LAMP. Indigenous bacteria were recovered from the 3-liter volumes of water at population sizes consistent with the number of pear or apple flowers added to the suspensions; the range was below detection for water only to 1.2×10^5 CFU/ml in 3-liter volumes to which 1,000 flowers had been added. For water only or water with flowers, LAMP reactions and dilution plates were negative for detection of *E. amylovora*. Strain Ea153N was always detected by LAMP assay from suspensions to which it had been amended regardless of the density of pear or apple flowers (Table 3). Ea153N also was recovered by dilution plating where counts of viable cells of CTT medium averaged 2.5×10^2 and 1.8×10^3 CFU/ml in the lower and higher pathogen-amended suspensions, respectively

Table 2. Primers designed for detection of *Erwinia amylovora* by loop-mediated isothermal DNA amplification targeted to a hypothetical protein region of plasmid pEA29^a

Primer name	Primer sequence (5' to 3')
Fip	TCGTGGTTATGCGATAACGCGTCAGGAAGTCCAGGGAGGTC
Bip	TGTGTACGATCCAGAGCACACGGTCATATGCAGGAGCAAGT
F	ACGCAAGCCTTCTAAAGCT
B	ATGGCCCGTGAAAAAGTCA
Loop	GGGGGAGAGTCCATTGGA

^a Primer concentrations in the reaction mix were 2.4 μM for Fip and Bip, 0.2 μM for F and B, and 0.4 μM for Loop. Melting temperatures for the primers were between 59 and 60°C. National Center for Biotechnology Information accession no. AF264948; bases 10,912 to 11,122 (22).

(Table 3). The presence of LAMP assay products was confirmed by electrophoresis on agarose gels (*data not shown*).

Single infested flower experiment. Single inoculated apple and pear flowers were colonized epiphytically by Ea153N at 4.1×10^6 to 7.1×10^7 CFU per flower (Table 4); these flowers were the source of pathogen in the 1.5-liter (2008) and 300-ml (2009) suspensions of water subjected to the LAMP assay. A positive LAMP assay resulted for all replications of a pathogen-infested pear or apple flower added to water only or to water amended with 100 pear or apple flower clusters (Table 4). LAMP assays and dilution plates for detection of *E. amylovora* were negative for water only and for washes of field-sampled pear or apple flowers only. Adding 100 flower clusters (approximately 600 flowers) to the wash water resulted in recovery of indigenous bacteria at population sizes that

ranged from 3.3×10^2 to 6.0×10^3 CFU per flower (*data not shown*).

Experimental orchard sampling. Flower clusters sampled at full bloom (2008 and 2009) and at petal fall (2008 only) from non-inoculated pear and apple orchards resulted in negative LAMP reactions (Table 5). Similarly, *E. amylovora* was not detected on dilution plates but indigenous bacteria were recovered from the floral washes at 2.5×10^4 to 1.6×10^6 CFU per flower (Table 5). In contrast, all 100-flower cluster samples from apple and pear orchards inoculated with Ea153N resulted in positive LAMP reactions (Table 5).

Commercial orchard survey. The 2008 sampling of commercial orchards in Oregon's Rogue Valley and Hood River Valley fruit-production districts was a first attempt to detect epiphytic *E. amylovora* without its artificial introduction onto pear and apple flowers. The pathogen was not detected by either LAMP or dilution-plate assay in the Rogue Valley (Table 6) but was detected by dilution plating of some samples from the Hood River Valley. For the Hood River Valley, 33% of samples from full bloom and petal fall samples were positive by dilution plating, with population size averaging 40 CFU per flower. Initially, all LAMP assays were negative but, after the DNA extracted from the 100-flower cluster samples was concentrated fourfold by SpeedVac evaporation, positive LAMP assays were obtained for the same samples that were positive by dilution plate. Consequently, in 2009 and 2010, the volume of water used to wash the 100-flower cluster samples was reduced from 1.5 liter (2008) to 0.3 liter.

In commercial orchards surveyed in 2009 and 2010, *E. amylovora* was detected by LAMP in 17% (120 of 714) of 100-flower cluster samples, and the frequency of detection again varied among orchard, production district, and year (Table 6). Overall, when detected by dilution plating, the population size of *E. amylovora* averaged 7.9×10^2 CFU per flower. Moreover, if the fire blight pathogen was detected by dilution plating (100 of 676 samples containing 100 flower clusters [14.7%]), it was also detected by LAMP (note: dilution-plating data were missing for 38 of the 714 samples). In contrast, for 11 flower cluster samples (1.6% of total samples), a positive LAMP was obtained but dilution plating was negative. The likelihood of detecting *E. amylovora* in 100-flower cluster samples also depended on the sample timing, with the mean incidence of positive LAMP assays approximately tripling at petal fall compared with midbloom (Table 6).

Although fire blight was observed by cooperators in 28 of 60 orchards, with severity classified as light (≤ 1 strike per tree) in 21 of 28 orchards, several orchards in the Hood River Valley in 2008 and the Columbia Basin and Utah in 2009 experienced significant epidemics. Overall, fire blight was observed in 20 orchards where *E. amylovora* was detected by LAMP during bloom, and LAMP detected *E. amylovora* in an additional 14 orchards where blight was not observed. Sixteen orchards were negative for LAMP and for fire blight. Of the eight orchards where LAMP did not detect the pathogen in a 100-flower cluster sample but a light severity of symptoms appeared, seven were central-Washington apple or-

Fig. 1. Endpoint absorbance readings of turbidity at optical density of 400 nm (OD₄₀₀) for loop-mediated isothermal DNA amplification assays performed on: **A**, a dilution series of cultured cells of *Erwinia amylovora* strain Ea153N boiled for 10 min after dilution and **B**, a dilution series of DNA extracted from *E. amylovora* Ea153N. Each data point is the mean of five replications; error bars represent $\pm$ one standard deviation of the mean.

Table 3. Percentage of positive loop-mediated isothermal DNA amplification (LAMP) assays as influenced by concentration of *Erwinia amylovora* strain Ea153N and density of pear of apple flowers in the wash suspension^a

Cultivar, conc. (CFU/ml) ^c	Positive LAMP assays (%) per flower density in 3-liter suspension of water ^b			
	0	10	100	1,000
Bartlett pear				
0	0	0	0	0
2.5×10^2	100	100	100	100
1.8×10^3	100	100	100	100
Gala Apple				
0	0	0	0	0
2.5×10^2	100	100	100	100
1.8×10^3	100	100	100	100

^a Field-grown flowers collected from experimental orchards located at the Botany and Plant Pathology Field Laboratory, Oregon State University, Corvallis.

^b Each mean is the average of five replications.

^c Concentration of viable Ea153N in suspension, determined by dilution plating the wash suspension onto CCT medium (9).

chards planted to cultivars (e.g., Pink Lady) prone to secondary bloom (5). For five of these orchards, samples were not taken at petal fall of the primary bloom period or during the secondary bloom.

For each year of sampling in commercial orchards, the comparison of positive and negative detection of *E. amylovora* in an orchard by LAMP with a weather-based fire-blight-forecasting model, CougarBlight, revealed that the outcome of the LAMP assay tended to segregate with the disease risk at the time of sampling (Fig. 2). For samples positive by LAMP, the CougarBlight 4-day degree-hour accumulation ($^{\circ}\text{C}$) averaged 144 ± 155 (standard deviation); degree-hour accumulations for samples negative by LAMP averaged 64 ± 143 .

Discussion

We developed a LAMP assay targeted to the pEA29 plasmid of *E. amylovora*, and evaluated the ability of this assay to detect the pathogen in washes of pear and apple flowers where it grows epiphytically as a prerequisite to infection of these hosts. Our guiding rationale for this effort was that information on the presence or absence of the fire blight pathogen in a local floral population could potentially improve tactical fire blight management and lessen destructive losses cause by this disease. Although the LAMP assay proved to be rapid, adequately sensitive, specific for our purpose, and relatively inexpensive and easy to perform, we were not fully convinced that sampling pear and apples flowers and shipping them for analysis by LAMP assay in a central laboratory facility is ready for widespread application. Nonetheless, the re-

sults of this study are useful for what they revealed about the epidemiology of fire blight within the Pacific Northwest, and for clarifying how molecular detection technologies and their delivery to stakeholders need to improve to achieve a practical test for the presence *E. amylovora* at the level of the individual orchard.

The LAMP assay, involving four to six primers, has proven to be robust when applied to environmental samples (Tables 3, 5, 6) (4,13,24,36). Less apparent is the effort that goes into the initial screening of LAMP primers. We designed and screened 45 primer sets (including some designed for chromosomal targets of *E. amylovora* not reported on here), which resulted in a substantial investment of time and materials as sets were tested and discarded. Overall, the pEA29 primers used in this study appeared to meet the criteria for specific and sensitive detection of *E. amylovora* from populations of pear and apple flowers. We chose primers targeted to the pEA29 plasmid because of its uniqueness to *E. amylovora* and its near ubiquity in strains of the pathogen sampled from the region. Occasionally, strains of *E. amylovora* that lack this plasmid have been isolated but they are generally rare (3 to 6% of isolates from the Pacific Northwest; V. O. Stockwell, unpublished data, 18). We developed a loop primer for the pEA29 primer set to shorten the time required to visualize a positive LAMP assay but, in specificity testing, we experienced possible mispriming with its use, particularly with the common epiphyte *P. agglomerans* (Table 1) (27,32).

Sensitivity of the LAMP assay decreased somewhat as the sample complexity increased. For example, 200 CFU/ml was the minimum density of pathogen cells from pure culture required to

Table 4. Percentage of positive loop-mediated isothermal DNA amplification (LAMP) assays from a single flower colonized by *Erwinia amylovora* strain Ea153N as influenced by presence or absence of pear or apple flowers in the wash suspension^a

Year, cultivar	Positive LAMP assays (%) per treatment added to water ^b			
	Water only	100-flower clusters	Single flower colonized by	
			Ea153N	Ea153N with 100-flower clusters
2008				
Bartlett pear	0	0	100 (7.3 $\pm$ 0.50)	100 (7.0 $\pm$ 0.71)
Gala apple	0	0	100 (6.8 $\pm$ 0.87)	100 (6.6 $\pm$ 1.07)
2009				
Bartlett pear	0	0	100 (6.6 $\pm$ 1.11)	100 (6.4 $\pm$ 0.56)
Gala apple	0	0	100 (6.4 $\pm$ 1.79)	100 (7.8 $\pm$ 0.17)

^a Stigmas on a newly opened, detached pear or apple flowers were inoculated with Ea153N and incubated for 3 to 4 days prior to the LAMP assay performed on the wash suspension. Field-grown flowers were collected from experimental orchards located at the Botany and Plant Pathology Field Laboratory, Oregon State University, Corvallis; 100-flower clusters per 0.3 (2009) or 1.5 liter (2008) volume were in a resealable plastic bag.

^b Three experimental replications each year. Log₁₀ population size (CFU per flower) and standard deviation of strain Ea153N on single flowers added to 1.5 (2008) or 0.3 (2009) liters of water. Pathogen population size was determined by dilution plating the wash suspension onto CCT medium (9).

Table 5. Results of loop-mediated isothermal DNA amplification (LAMP) assays performed on 100-flower cluster samples from experimental pear and apple orchards located near Corvallis, OR, which were inoculated or not inoculated with *Erwinia amylovora*^a

Year, orchard cultivar	Inoculated ^c	Positive/total ^d	Population size log ₁₀ (CFU/flower) ^b	
			<i>E. amylovora</i>	Total bacteria
2008				
Bartlett pear	No	0 of 6	Not detected	5.6 $\pm$ 0.10
Fuji apple	No	0 of 6	Not detected	4.7 $\pm$ 0.29
Jonathon apple	No	0 of 6	Not detected	4.6 $\pm$ 0.15
Bartlett pear	Yes	6 of 6	2.7 $\pm$ 0.70	5.2 $\pm$ 0.30
Gala apple	Yes	6 of 6	3.7 $\pm$ 0.53	4.4 $\pm$ 0.24
Golden Delicious apple	Yes	6 of 6	4.5 $\pm$ 0.16	5.0 $\pm$ 0.28
2009				
Bartlett pear	No	0 of 3	Not detected	4.4 $\pm$ 0.41
Mixed pear	No	0 of 3	Not detected	4.7 $\pm$ 0.16
Braeburn apple	No	0 of 3	Not detected	4.7 $\pm$ 0.08
Bartlett pear	Yes	3 of 3	3.8 $\pm$ 0.07	6.2 $\pm$ 0.24
Gala apple	Yes	3 of 3	3.6 $\pm$ 0.05	5.4 $\pm$ 0.05

^a Orchards were located at the Oregon State University, Botany and Plant Pathology Field Laboratory; 100-flower cluster samples (approximately 600 flowers) were suspended in 1.5 (2008) or 0.3 liter (2009) volumes of water.

^b *E. amylovora* population size was determined by dilution plating the wash suspension onto CCT medium (9) and total bacterial population size was determined by dilution plating the wash suspension onto Pseudomonas agar F. Population size $\pm$ standard deviation among 100-flower cluster samples.

^c Indicates if trees in orchards were mist inoculated with a suspension of strain Ea153N at full bloom.

^d Number of positive LAMP assays of total samples.

visualize a positive LAMP assay without aid of a spectrophotometer. In contrast, the first efforts with Ea153N in suspension with flowers failed to detect the pathogen at 250 CFU/ml (*data not shown*). This prompted the use of the intermediate concentration step, where a 15-ml subsample of the prefiltered wash was embedded on 0.2-µm membrane, resulting in detection of the pathogen at 2.5×10^2 CFU/ml regardless of floral density (Table 3). Similarly, several 2008 samples from commercial orchards in the Hood River Valley resulted in detection by dilution plate but not by LAMP (Table 6); this prompted the reduction in the floral wash volume from 1.5 to 0.3 liters. In the 2009 and 2010 commercial orchard surveys, use of the 0.3-liter wash volume with the embedded filter concentration step resulted in similar rates of pathogen detection and high sample congruence between the LAMP and dilution plating methods. Therefore, we concluded that the LAMP protocol has a detection limit of approximately 10^3 CFU/ml when 300 ml of water is used to wash the 100-flower cluster sample, and approximately 10^2 CFU/ml when 15 ml of the floral wash is reduced to 1 ml by filter concentration.

We used a heuristic approach to determine the number of flower clusters to include in a sample and the number of samples to make in each orchard, mostly because we had little prior knowledge of the size of epiphytic populations of *E. amylovora* in commercial orchards across the region. We required an orchard sample that would be large enough to detect a potentially significant (disease-causing) population of *E. amylovora* but simple enough for a cooperator in the field to understand and implement. In a study with a similar objective, Kritzman et al. (15) used preliminary sampling from two orchards and statistical theory to examine the problem of sample size for detection of epiphytic *E. amylovora* on individual flowers. The conditions of their study, however, differed greatly from ours in that their optimal sample size determination was based on orchards where 10 to 40% of individual flowers were

colonized by *E. amylovora*, and 15 to 20% of flower clusters became diseased. This high level of pathogen infestation led to the conclusion that a 20-flower sample from a few trees was sufficient to achieve an accuracy level within 10% of the true mean (15). In contrast, we detected the pathogen in only 130 (16%) of 788 100-flower cluster samples (600 flowers) from 60 commercial orchards and, from data provided by cooperators, we estimated the mean observed incidence of diseased flower clusters to be in the range of 0.01 to 0.1%. For our study, if a hectare from which a positive LAMP assay can be obtained is assumed to be randomly distributed (a 100-flower cluster sample represented approximately one hectare) then, from binomial probability tables (6), the probability of not detecting *E. amylovora* is 7.5% when the true mean is 0.16 (16% of hectares have a detectable epiphytic *E. amylovora* population) and 15 LAMP assays are conducted per 5-ha orchard. The distribution of positive LAMP samples, however, likely deviates somewhat from random (Table 6) (15). Although it is beyond the scope of this study, the variability of epiphytic *E. amylovora* populations and potential for aggregation among samples requires further investigation relative to factors that appear to influence the probability of a positive sample: individual fruit-production district (Table 6), the bloom stage at the time of sampling (Table 6), the disease history of an orchard (30), and the weather-based prediction of fire blight risk (Fig. 2).

Heat unit accumulation models (e.g., Cougarblight; 30) that identify when conditions are favorable for *E. amylovora* to grow on and disperse among pome fruit flowers are the principal tool used by growers and advisors to determine the need for protective antibiotic sprays (30). Consequently, it was expected that orchards testing positive by LAMP assay on a given sampling date (defined as at least one positive sample of 100 flower clusters per orchard) would tend to be associated with periods of higher disease risk (Fig. 2). More interesting were the many negative LAMP assays

Table 6. Summary of loop-mediated isothermal DNA amplification (LAMP) assays performed to detect *Erwinia amylovora* in 100-flower cluster samples collected from commercial pear and apple orchards in the Pacific Northwest region of the United States from 2008 to 2010^a

Year, state	Production area	Host	No. of orchards	No. of positive LAMP assays of total 100-flower cluster samples ^b			Isolation on media ^c	CFU per flower ^d	No. with fire blight ^e	Severity ^f
				Mid-bloom	Full bloom	Petal fall				
2008										
Oregon	Rogue Valley	Pear	3	0 of 15	0 of 14	—	No	—	0	—
	Hood River Valley	Pear	3	0 of 15	3 ^g of 15	7 ^g of 15	Yes	1.6	2	Light to mod
2009										
Oregon	Rogue Valley	Pear	3	3 of 20	0 of 20	2 of 20	Yes	3.3	1	Light
	Hood River Valley	Pear	6	6 of 30	6 of 30	7 of 25	Yes	3.3	2	Light
	Hood River Valley	Apple	2	0 of 8	2 of 8	4 of 8	Yes	2.2	1	Light
	Walla Walla Valley	Apple	4	0 of 20	4 of 20	11 of 20	Yes	3.3	3	Light
California	Lake County	Pear	4	2 of 15	2 of 15	1 of 15	Yes	1.2	1	Light
Washington	Okanogan Valley	Pear	1	0 of 4	0 of 6	2 of 4	Yes	3.8	1	Light
	Wenatchee Valley	Pear	2	0 of 10	0 of 10	0 of 10	No	—	0	—
	Columbia Basin	Apple	3	0 of 15	0 of 15	0 of 10	No	—	3	Light to mod
Utah	Utah County	Apple	6	11 of 19 ^h	19 of 25 ^h	10 of 18 ^h	Yes	3.4	7	Mod to heavy
2010										
Oregon	Rogue Valley	Pear	2	0 of 12	0 of 12	0 of 12	No	1.5	0	—
California	Sutter County	Pear	6	4 of 30	0 of 30	0 of 30	Yes	2.0	0	—
California	Lake County	Pear	5	0 of 30	0 of 30	20 of 40	Yes	—	0	—
Washington	Okanogan Valley	Pear	1	2 of 3	0 of 5	—	No	—	1	Light
	Yakima Valley	Apple	9	0 of 30	2 of 30	—	Yes	1.6	6	Light
Summary	...	...	60	28 of 276 (10%)	38 of 285 (13%)	64 of 227 (28%)	...	2.8	28	...

^a The 100-flower cluster samples (approximately 600 flowers per sample) were washed in 1.5 liter (2008) or 0.3 (2009 and 2010) of water in a resealable plastic bag.

^b Number of positive LAMP assays of total 100-flower cluster samples; — indicates not sampled.

^c Indicates if *E. amylovora* was recovered from the washes of 100-flower cluster samples after dilution plating onto CCT medium (9).

^d Average log₁₀ population size of *E. amylovora* (CFU per flower) recovered from the wash of 100-flower cluster samples.

^e Number of orchards with fire blight.

^f Disease severity in orchards with fire blight. Range of disease ratings applied to the orchards: light = 1 strike per tree, moderate (mod) = 2 to 5 strikes per tree, and heavy ≥ 6 strikes per tree.

^g Positive detection by LAMP required a fourfold speedvac concentration of extracted DNA.

^h In Utah, one 100-flower cluster sample was collected from each orchard daily over a period to 10 to 12 days; dates were grouped into approximate stage of bloom.

(163 of 195) obtained for the 100-flower cluster samples during periods when the weather-based model, Cougarblight, predicted at least moderate risk (index values >225). Similarly, 83 of 121 positive LAMP assays occurred on days when the CougarBlight index was below moderate risk although, in most cases, these samples were from orchards with a recent history of fire blight and, in some cases, the risk index had been at or above “moderate risk” shortly before the sample date. These inconsistencies relate back to our guiding rationale, which was that fire blight management could be refined and improved if variation in epiphytic populations of the pathogen could be readily measured. To this end, cooperating growers provided positive feedback on the value of information from the LAMP assay, with several reporting that a positive assay result, especially early in bloom, caused an intensification of their fire blight control efforts. In contrast, one cooperating pear grower used information from LAMP assays to withhold a protective spray (correctly) after negative results were weighed with the current risk of disease as determined by a forecasting model.

Over the period of this study, in part because we interacted with numerous individuals who made day-to-day decisions on disease control, we became more confident that a method of knowing the

Fig. 2. Seasonal summaries of weather-based assessments of fire blight risk (Cougarblight model; 30) in relation to detection of *Erwinia amylovora* by loop-mediated isothermal amplification (LAMP) assay of washes of pear and apple flowers sampled from commercial orchards in the Pacific Northwest region of the United States. Open arrows indicate no detection of *E. amylovora* by LAMP on the corresponding date, and solid arrows indicate that at least one LAMP assay performed on a 100-flower cluster sample was positive for *E. amylovora*. Cougarblight provides a 4-day moving sum of degree hours >15.5°C (60°F); values of this index are used to assess the likelihood that epiphytic populations of *E. amylovora* have developed in pear and apple flowers (30). Index values shown in each panel are a composite from temperature data (see Materials and Methods) measured at each of production areas sampled that season: **A**, 2008; Rogue and Hood River Valleys of Oregon; **B**, 2009, Rogue and Hood River Valleys of Oregon, Lake County, CA, and Yakima, Wenatchee, and Okanogan Valleys of Washington; and **C**, 2010, Rogue Valley of Oregon, Lake and Sutter Counties, CA, and Yakima and Okanogan Valleys of Washington.

presence or absence of the pathogen (a LAMP assay or a detection technology of similar sensitivity) could provide information useful for management of fire blight. We were unconvinced, however, that a centralized laboratory facility was the best model for providing this service to orchard managers. We (including cooperators) experienced several logistical problems stemming from within-district dynamics of pear and apple bloom, the rapid development of epiphytic populations of *E. amylovora* when temperature conditions were favorable, and the requirements for routine sampling of flowers, overnight shipping, and quick dissemination of LAMP results. Although equipment needs for LAMP assays are minimal, performing the assay requires personnel with excellent laboratory skills and a thorough understanding of the risk and prevention of molecular contamination (4). With good laboratory organization, we were able to process dozens of orchard samples simultaneously, and began and completed the floral washes and LAMP assays over a period of a few hours. Time for preparation and other fixed costs, however, such as those that would enable an efficient response over the relatively short period of pear and apple bloom, may discourage the involvement of for-profit service providers but perhaps not a grower-supported, regional extension facility. More promising are expected technological advances that offer the potential for point-of-care (i.e., on-site; 14) detection by nonprofessionals, such as the recent adaptation of LAMP to lateral-flow devices (36). In this regard, recent reviews (14,17) make the case that future improvements in plant and veterinary disease diagnosis will depend on increased deployment of detection technologies “close to the suspect case of disease away from dedicated laboratory facilities” (14). With further development of pathogen-detection technologies for use on-site, the results of our study should prove useful to practitioners seeking to implement an early detection protocol for *E. amylovora*.

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