Detection of *Erwinia amylovora* by Nested PCR and PCR-Dot-Blot and Reverse-Blot Hybridizations

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ABSTRACT

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The sensitivity and specificity of four methods based on the polymerase chain reaction (PCR) for detection of *Erwinia amylovora* were compared. A previously developed single-round PCR assay was used to amplify a specific 1-kb DNA fragment of plasmid pEA29, known to be unique to and conserved in *E. amylovora*. The ends of the 1-kb single-round PCR product were sequenced, and two new oligonucleotide primers were designed from sequences internal to the original primers and used in nested PCR. These primers directed amplification of a 844-bp fragment from the 1-kb first-round PCR product directly from *E. amylovora* cells but not from other bacteria associated with apple. Less than one cell of *E. amylovora* from pure culture was detected with nested PCR—an increase in sensitivity of 1,000-fold compared to single-round PCR. The lower limit of detection of PCR-dot-blot and reverse-blot hybridization methods was approximately 20 cells. Weak positive signals were sometimes produced by bacteria other than *E. amylovora* in hybridization experiments but only if nonspecific PCR products were visible on ethidium bromide-stained agarose gels. When asymptomatic apple tissue was screened, *E. amylovora* was detected in 61, 84, and 100% of leaf samples; 0, 66, and 80% of axillary bud samples; and 4, 27, and 75% of mature fruit calyx samples by first-round PCR, PCR-dot-blot hybridization, and nested PCR, respectively. The potential applications of each method are discussed.

Fire blight, caused by *Erwinia amylovora*, is the most destructive bacterial disease affecting apple (*Malus* × *domestica* Borkh.), pear (*Pyrus communis* L.), and several rosaceous ornamentals. Fire blight has been reported throughout the apple- and pear-producing regions of North America, Europe, western Asia, and New Zealand but has not been documented in eastern Asia, South America, Australia, or South Africa (30). The pathogen can survive as an endophyte (10,31), an epiphyte (17,26,28), or in latent infections (22). The movement of infested or infected asymptomatic plants or plant parts could serve as a means of introducing this serious disease into other geographic regions. In some countries where fire blight does not occur, *E. amylovora* is a regulated organism, and trade of nursery stock and fruit is restricted. Methods of detecting *E. amylovora* in nursery stock, budwood, and fruit are needed to prevent the introduction of the pathogen into fire blight-free regions and to limit the spread of new strains among regions where fire blight currently exists.

Nurseries and plant health inspection agencies would both benefit from the development of more specific and sensitive procedures for routinely indexing propagation stock and plant tissue from *E. amylovora* before symptoms are apparent. A polymerase chain reaction (PCR) method based on the amplification of a 1-kb fragment of pEA29 (3), a plasmid common in all strains of *E. amylovora* (6,12), has been used to identify colonies of *E. amylovora* and to detect *E. amylovora* in inoculated apple seedlings (3) and extracts from typical fire blight lesions (P. S. McManus, *unpublished data*). The PCR method proved more sensitive than conventional plating methods for detecting *E. amylovora* on asymptomatic apple leaves (16). However, the sensitivity of the method was inversely related to the level of apple tissue extract in the PCR reaction mixture (P. S. McManus, *unpublished data*). Many plant tissues harbor inhibitors of PCR (8,13,18,19,23), which can result in false negatives, especially when tissue is tested directly without DNA purification.

Modifications of PCR and the development of procedures that combine PCR and DNA hybridization techniques permit even greater specificity and sensitivity in detecting microorganisms. In nested PCR, sensitivity and specificity of detection are enhanced by performing a second round of PCR with amplified DNA from first-round PCR as the template and primers internal to the first-round primers (20,25). In PCR-dot-blot hybridization, PCR products are immobilized on a membrane and hybridized to a labeled DNA probe. In reverse-blot hybridization, the label is incorporated into PCR products during amplification, and the labeled products are hybridized to an unlabeled DNA probe immobilized on a membrane (9). The potential for widespread application of these molecular techniques in diagnosing plant pathogens is great.

In the present study, we describe nested PCR, PCR-dot-blot hybridization, and reverse-blot hybridization assays for detecting *E. amylovora*. We define the limits of sensitivity of the methods in detecting *E. amylovora* from pure culture and in apple-tissue extract. By comparing our methods with an earlier PCR-based method (3), we demonstrate increased sensitivity and versatility of PCR in the detection of *E. amylovora* associated with apple tissues.

MATERIALS AND METHODS

Bacterial strains and detection limits. Strains of bacteria and their sources are listed in Table 1. Bacteria were incubated (37°C for *Escherichia coli*, 24°C for all others) with shaking for 16 h in Luria-Bertani (LB) medium (24). Cells were pelleted, washed

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with water, and adjusted to 1 to 5 × 10^8 colony forming units (CFU) per ml (OD_{640nm} = 0.14), determined by plating dilutions onto King's medium B (11) or LB agar. Dilution series were prepared in water or apple-leaf homogenate (1 g of healthy leaf tissue per 10 ml of water), and 1 µl of each dilution was used for PCR as described below. Detection threshold experiments were performed four times.

Preparation of apple tissue for PCR. Sterile distilled water and new plastic- and glassware and blades were used whenever possible during sample processing. Forceps, scalpels, spatulas, and other materials were routinely treated with 1 M HCl, 2% sodium hypochlorite, and autoclaving to destroy contaminating DNA. Shoots (approximately 50 cm long) from various cultivars of apple were collected from trees in a scion orchard during mid-August 1994 when workers were collecting budsticks for grafting. No symptoms were visible at the time of collection, but fire blight infections had been pruned from some of the scion trees in previous years. Symptomless shoots also were collected from trees with fire blight in an orchard at Michigan State University (MSU) in East Lansing. For each shoot, approximately 10 g of leaf tissue (8 to 10 leaves) was suspended in 50 ml of water and was washed for 1 h at 200 rpm on a rotary shaker. Wash solutions were filtered (Whatman No. 1), and centrifuged for 15 min at 8,000 × *g*; individual pellets were resuspended in 100 µl of water. Defoliated shoots were swabbed with 70% ethanol. Ten axillary buds were cut from each shoot, held on a glass microscope slide with forceps, and sliced into 1- to 2-mm-thick sections using a new razor blade and slide for each shoot. Bud sections from each shoot were combined and washed in 2 ml of water for 1 h at 200 rpm on a rotary shaker. Apple fruit (cultivar Jonathan) were collected from the MSU orchard during October 1994. Because *E. amylovora* was more frequently isolated from the calyx than from other surfaces of apple fruit (7), the calyx and other remnant flower parts were cut from each fruit using a scalpel. Calyces were individually macerated in 2 ml of water with a metal spatula. For positive controls, effluents from leaf-wash solutions, bud sections, or macerated calyx tissue were spiked with 5 × 10^5 CFU of *E. amylovora*. For negative controls, spatulas or scalpels were dipped into effluents from leaf-wash solutions, bud sections, or macerated calyx tissue that previously had been determined to be free of *E. amylovora*. Bud, calyx, and control samples were filtered through two layers of cheesecloth and centrifuged for 1 min at 13,000 × *g*; individual pellets were resuspended in 100 µl of water.

Primers and sequencing of the PCR product. All primers were synthesized with a 380B DNA synthesizer (Applied Biosystems, Inc., Foster City, CA) at the Macromolecular Facility, Department of Biochemistry, MSU. Primers A (5′-CGGTTTTTAACGCTGGG-3′) and B (5′-GGGCAAATACTCGGATT-3′) (3) were used in single- and first-round PCR. Automated fluorescent sequencing of the amplified product from first-round PCR (using strain CA11 of *E. amylovora*) was performed using primers A and B, the ABI (Applied Biosystems, Inc.) catalyst for *Taq* cycle sequencing, and the ABI 373A sequencer for analysis of the product at the MSU Department of Energy-Plant Research Laboratory Plant Biochemistry Facility. For nested PCR, new primers, AJ75 (5′-CGTATTCACGGCTTCGCAGAT-3′) and AJ76 (5′-ACCCGCCAGGATAGTCGCATA-3′), were selected from sequences internal to primers A and B. Additional internal sequence of the first-round PCR product, including the entire sequence of the nested PCR product, was obtained using primers AJ75 and AJ76 for automated sequencing.

First-round and nested PCR. The PCR reaction mixture (50 µl) contained (final concentrations) 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 8 µg of bovine serum albumin, 5% dimethyl sulfoxide, 0.1% Tween 20, 150 µM each dATP, dCTP, dGTP, and dTTP, 50 pmol of each primer of the appropriate pair, and 2.5 units of *Taq* DNA polymerase (Gibco BRL, Gaithersburg, MD). DNA was denatured in a programmable thermal controller (PTC-150, MJ Research, Inc., Watertown, MA) at 94°C for 5 min followed by 37 cycles of denaturation for 1 min, annealing at 52°C for 2 min, and polymerization at 72°C for 2 min. Final DNA extension at 72°C for 15 min was followed by a soak at 4°C.

For first-round PCR, 5 µl of each tissue or control sample was mixed gently with 5 µl of GeneReleaser (BioVentures, Inc., Murfreesboro, TN) in a 0.5-ml centrifuge tube. The mixture was overlaid with light mineral oil, capped, heated for 5 min at full power in a 900-

TABLE 1. Bacteria tested and size of products obtained with single-round and nested polymerase chain reaction (PCR)[a]

Species Strain	Reference or source	Single-round PCR A & B 1 kb	Single-round PCR A & B Other	Single-round PCR AJ75 & AJ76 0.8 kb	Single-round PCR AJ75 & AJ76 Other	Nested PCR A & B; AJ75 & AJ76 0.8 kb	Nested PCR A & B; AJ75 & AJ76 Other
Erwinia amylovora							
EL01	5	+	–	+	–	+	–
CAll	5	+	–	+	–	+	–
BCN20	15	+	–	+	–	+	–
RN8	15	+	–	+	–	+	–
DM5	Apple; Mich.	+	–	+	–	ND[b]	ND
R-KK4	Raspberry; Mich.	+	–	+	–	+	–
E. herbicola							
BC9	15	–	+	–	–	–	–
6a	27	–	+	–	–	–	–
252	29	–	+	–	–	–	–
351	29	–	+	–	–	–	–
112Y	29	–	+	–	–	–	–
Pseudomonas syringae pv. *papulans*							
Psp32	4	–	+	–	–	–	–
Psp36	4	–	+	–	–	–	–
Escherichia coli							
JM109	Promega, Corp.	–	+	–	–	ND	ND
Miscellaneous strains[c]	Apple; Mich.	–	+	–	–	–	–

[a] Single-round PCR was performed with outer primers A and B or internal primers AJ75 and AJ76. Nested PCR was performed with primers A and B for the first round and primers AJ75 and AJ76 for the second round. Reactions contained 10^4–10^5 colony forming units of bacteria. Presence or absence of PCR products (1 kb or other sizes) is indicated by + and –, respectively.

[b] ND = not determined.

[c] Six unidentified bacterial isolates were tested by single-round PCR with each primer set. Four of the six isolates were tested by nested PCR. Results were similar for all isolates.

W microwave (Sears Kenmore model 565.8721481), and incubated at 80°C for 5 min in the thermal controller. PCR reaction mixture (40 µl) containing primers A and B was added to each sample, and the amplification steps were initiated. For nested PCR, 1 µl of first-round product was added to 49 µl of PCR reaction mixture containing primers AJ75 and AJ76. Water (1 µl) was used as a negative control for the nested PCR. Samples were overlaid with light mineral oil and subjected to amplification. Aliquots of PCR products (20 µl) were analyzed by electrophoresis through 1% agarose gels. PCR assays were performed twice for each tissue sample.

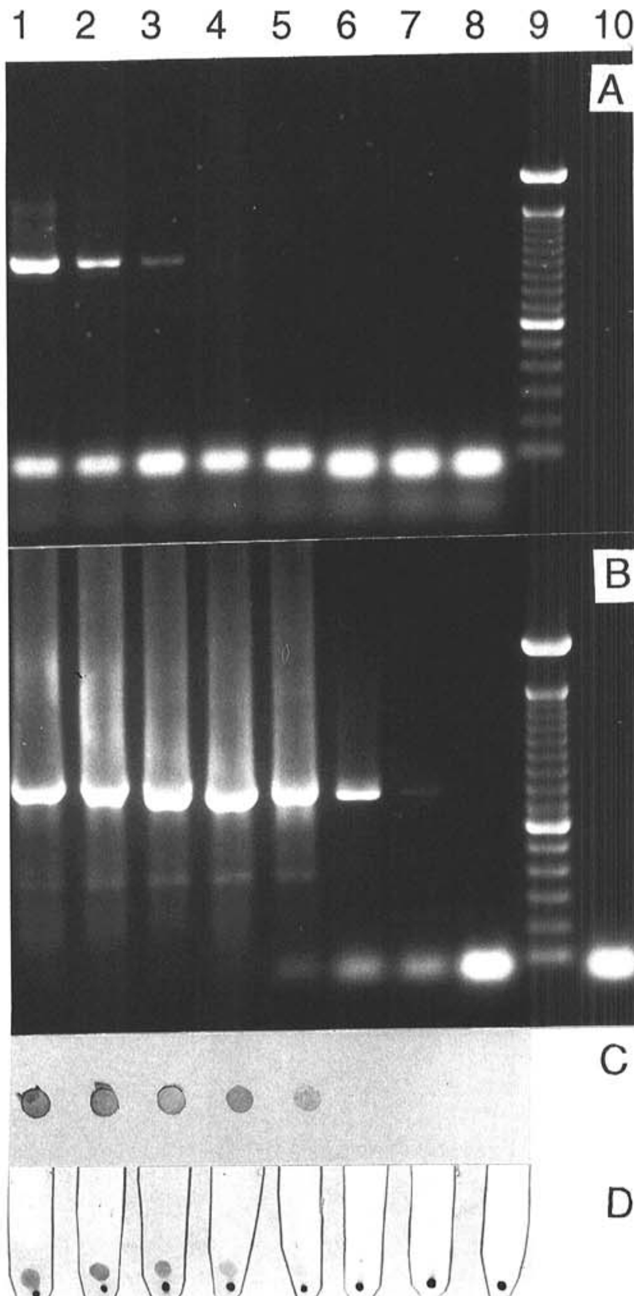

Fig. 1. Sensitivity of assays for detection of *Erwinia amylovora* strain EL01 in water. Number of colony forming units: lane 1, 2×10^5; lane 2, 2×10^4; lane 3, 2×10^3; lane 4, 2×10^2; lane 5, 2×10^1; lane 6, 2×10^0; lane 7, 2×10^{-1}; and lane 8, 0. Lane 9, 100-bp ladder. Lane 10, **A,** empty lane; **B,** water negative control for nested PCR. **A,** First-round polymerase chain reaction (PCR) products amplified from primers A and B. **B,** Nested PCR products of DNA from **A** amplified with internal primers AJ75 and AJ76. **C,** Dot-blot of PCR products from **A** hybridized to digoxigenin-labeled probe N844. **D,** Reverse-blot hybridization. Digoxigenin-11-UTP was incorporated into PCR products amplified from primers A and B; labeled products were hybridized to probe N844, which had been immobilized on strips of nylon membrane; the small dots near the tips of the detection strips were applied as reference points for spotting N844 DNA. PCR products in **A** and **B** were separated by electrophoresis through 1% agarose gels.

Dot-blot hybridization. First-round PCR products (20 µl) were incubated in 80 µl of denaturation solution (2 N NaOH, 50 mM EDTA) for 10 min and transferred to nylon membranes (GeneScreen Plus, Dupont NEN Research Products, Boston) with a Bio-Dot dot-blotting apparatus (Biorad Laboratories, Inc., Hercules, CA). The 844-bp nested PCR product, designated N844, was recovered from an agarose gel by elution onto DEAE-cellulose paper (24) and labeled with digoxigenin-11-dUTP using the Genius DNA labeling and detection kit (Boehringer Mannheim Corp., Indianapolis, IN). Prehybridization, hybridization, and chemiluminescent or colorimetric detection were performed according to the manufacturer's instructions.

Reverse-blot hybridization. The 844-bp nested PCR product (N844) was pipetted in 0.5-µl aliquots onto a nylon membrane near dots of indelible ink serving as reference points. The membrane was floated on 0.5 N NaOH for 4 min to denature the DNA, blotted briefly on a paper towel, and floated on 1 M Tris-HCl (pH 8) for 4 min to neutralize the DNA. The membrane was allowed to air-dry and was cut into 8-mm-wide × 30-mm-long detection strips, each strip with a single 500-ng spot of N844 DNA. Individual detection strips were incubated at 68°C for 15 min in 1 ml of Genius prehybridization buffer in 1.7-ml centrifuge tubes. First-round PCR was performed as described above, except the deoxynucleotide concentrations were 200 µM each dATP, dCTP, and dGTP, 190 µM dTTP, and 10 µM digoxigenin-11-dUTP. The digoxigenin-labeled PCR products were denatured at 100°C for 5 min, cooled on ice, and 10 µl of each was added to tubes containing prehybridization buffer and detection strips. After hybridization for 1 h, detection strips were shaken for 5 min at room temperature in 2X wash solution (prepared according to Genius kit instructions) and for 10 min at 68°C in 0.5X wash solution. Colorimetric detection was performed according to the Genius kit instructions, except that blocking of detection strips was done for 10 min and incubation with alkaline phosphatase conjugated anti-digoxigenin antibody was done for 15 min, followed by two 10-min washes in maleate buffer (100 mM maleic acid [pH 7.5], 150 mM NaCl).

Specificity of the assays. The specificity of primers A and B and of primers AJ75 and AJ76 was tested by performing PCR for 37 cycles with 10^4 to 10^5 CFU of each bacterial strain (Table 1). To test the specificity of nested PCR, first-round PCR with primers A and B was followed by 37 cycles of PCR with primers AJ75 and AJ76. The specificity of probe N844 and the detection strips was tested by hybridization to dot-blots of first-round PCR products and to digoxigenin-labeled first-round PCR products of the various bacteria, respectively.

RESULTS AND DISCUSSION

The 844-bp sequence of the PCR product obtained with primers AJ75 and AJ76 plus 45- and 111-bp flanking sequences were submitted to GenBank (accession number U19254). The flanking sequences were interior to the two 17-mer oligonucleotides (primers A and B) used for first-round PCR and for direct sequencing of the first-round product. Although primers A and B were reportedly derived from a 0.9-kb *Pst*I fragment (2,3), the amplified fragment from *E. amylovora* strain CA11 was slightly larger than 1 kb based on our electrophoresis and sequencing data. Moreover, a cloned and sequenced 0.9-kb *Pst*I fragment of strain CA11 lacked sequences for primers A and B and did not hybridize with probe N844 (P. S. McManus, *unpublished data*). These results indicate that the PCR product of primers A and B was likely a portion of the 1.1-kb *Pst*I fragment F rather than the 0.9-kb *Pst*I fragment G of pEA29 (2,6). Whether fragment F or G was ampli-

fied does not affect the usefulness of these primers for identification of *E. amylovora*, but this information may be important in future studies of pEA29.

With first-round PCR, as few as 200 cells of *E. amylovora* grown in LB medium and serially diluted in water were detected (Fig. 1A). With PCR-dot-blot (Fig. 1C) and reverse-blot hybridizations (Fig. 1D), as few as 20 cells were detected. With nested PCR, the level of detection was less than one cell (Fig. 1B). Because dead or nonculturable cells probably were present in the dilutions, plate counts likely underestimated the number of cells. Also, more than one cell may have comprised some CFU. Direct counts of cells, as opposed to culturable counts, would provide a more accurate measure of the sensitivity of the assays (21).

When *E. amylovora* was diluted in apple-tissue homogenate, detection sensitivity was 100 to 1,000 cells using first-round PCR, about one-fifth the sensitivity in water (results not shown). Levy et al. (13) used the commercial product GeneReleaser to prepare intractable plant tissues for PCR detection of viruses, viroids, and mycoplasma-like organisms. Using GeneReleaser and "hot start" PCR according to the manufacturer's protocol, detection sensitivity in apple-tissue homogenate was increased five- to tenfold. Thus, the sensitivity of detection in tissue extract approached the sensitivity of detection in water, while time-consuming and laborious DNA extraction and purification methods were avoided.

E. amylovora was detected in 61, 84, and 100% of leaf; 0, 66, and 80% of axillary bud; and 4, 27, and 75% of calyx samples with first-round PCR, PCR-dot-blot hybridization, and nested PCR, respectively (Table 2). Reverse-blot hybridization was not performed with all the asymptomatic tissue samples because the method is inconvenient when the sample size is large. Invariably, the order of sensitivity from least to most sensitive was: first-round PCR, PCR-dot-blot hybridization, and nested PCR. Any sample positive by first-round PCR was also positive in the other methods. Occasionally southern blots of nested PCR products were probed with the 1-kb first-round product to confirm that the 0.8-kb product originated from *E. amylovora*. The sensitivity of nested PCR was striking; the 844-bp nested PCR product was often clearly visible even when no first-round product was detected in agarose gels (Figs. 2 and 3). The incidence of detection of *E. amylovora* was greater with asymptomatic samples from blighted trees at MSU than with samples from the apparently healthy trees at the scion orchard. The epiphytic and endophytic existence of *E. amylovora* in asymptomatic tissue collected from or near trees with fire blight has been documented (7,10,17,26, 28,31). However, *E. amylovora* has rarely been recovered from tissue collected in a fire blight-free setting (10,31). It is significant that we frequently detected *E. amylovora* associated with tissue from a scion orchard in which fire blight had been absent during the current year and rare in previous years.

Previously we were unable to detect the pathogen in buds and in only 16% of leaf samples from trees in the scion orchard using single-round PCR (16). The improved sensitivity of nested PCR compared to first-round PCR in detecting *E. amylovora* associated with asymptomatic apple tissue was probably due in part to dilution of plant-derived inhibitors of PCR and nonspecific DNA prior to the second round of amplification. Also, primer binding almost certainly would be more efficient in nested PCR than in first-round PCR because the target DNA is located on the 1-kb linear, first-round PCR product rather than on the 29-kb circular, super-coiled plasmid encased within bacterial cells.

When bacteria other than *E. amylovora* were subjected to PCR with primers A and B, amplification products of various sizes were observed (Table 1; Fig. 4) as previously reported by Bereswill et al. (3). However, these nonspecific products were readily distinguished from the single 1-kb product indicative of *E. amylovora* (Fig. 4). When nonspecific bands and low molecular-weight products, possibly primer multimers, were visible on agarose gels, weak positive signals were sometimes detected by PCR-dot-blot and reverse-blot hybridizations (Fig. 4). False posi-

TABLE 2. Comparison of three polymerase chain reaction (PCR)-based methods for detecting *Erwinia amylovora* in symptomless apple tissue

Tissue[a] Location[b]	No. of samples	Incidence of *E. amylovora* detection by PCR (%)		
		First-round[c]	Dot-blot[d]	Nested[e]
Leaf				
Scion orchard	26	39	81	100
MSU orchard	28	82	86	100
Total	54	61	84	100
Bud				
Scion orchard	26	0	62[f]	73
MSU orchard	52	0	70[g]	87
Total	78	0	66	80
Calyx				
MSU orchard	48	4	27	75

[a] Samples (10 g) of leaf tissue (8–10 leaves) were shaken in 50 ml of water for 1 h. Buds were sliced into 1- to 2-mm-thick sections. Sections from 10 buds per budstick were combined and shaken in 2 ml of water for 1 h. Calyces were cut from mature apple fruit and individually macerated in 2 ml of water. Samples were filtered, concentrated by centrifugation, and resuspended in 100 µl of water.

[b] Samples were collected from a fire blight-free scion orchard at a commercial nursery and from an orchard with fire blight at Michigan State University (MSU), East Lansing.

[c] Aliquots (5 µl) of each sample were used in single-round PCR with primers A and B.

[d] DNA in 20-µl aliquots of single-round PCR products was denatured, neutralized, applied to a nylon membrane, and hybridized with probe N844.

[e] Aliquots (1 µl) of single-round PCR products were used in a second round of PCR with primers AJ75 and AJ76.

[f] Number of samples was 13.

[g] Number of samples was 39.

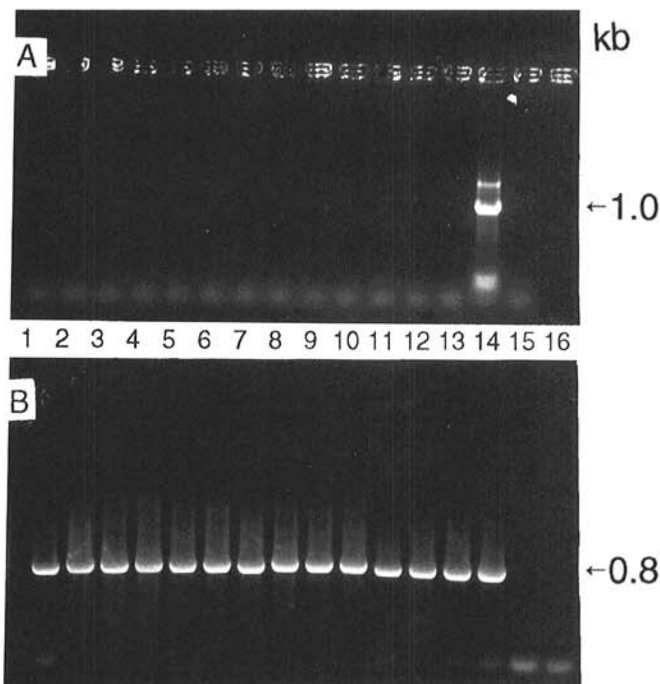

Fig. 2. Comparison of assays for detection of *Erwinia amylovora* in symptomless axillary buds of apple from an orchard with fire blight at Michigan State University, East Lansing. **A,** First-round polymerase chain reaction (PCR) products amplified from primers A and B. **B,** Nested PCR products of DNA from **A** amplified with internal primers AJ75 and AJ76. Lanes 1–13, bulked samples of 10 buds per sample; lane 14, bud rinse spiked with 5×10^5 colony forming units of *E. amylovora*; lane 15, bud rinse previously determined to be free of *E. amylovora*; and lane 16, **A,** empty lane; **B,** water negative control for nested PCR. PCR products were separated by electrophoresis through 1% agarose gels.

tive results might be prevented by using only AJ75, AJ76, or some other portion of N844 as a probe. No amplification products arose from bacteria other than *E. amylovora* after single-round PCR with primers AJ75 and AJ76 (Table 1), indicating that primers AJ75 and AJ76 may be more specific and perhaps better suited for single-round PCR than primers A and B. However, the relative sensitivity of the two primer sets has not been established. Amplification products from bacteria other than *E. amylovora* were not detected with nested PCR, even when nonspecific bands were visible after first-round PCR (Table 1). Thus, in addition to extreme sensitivity, nested PCR correctly identified *E. amylovora* and distinguished it from other bacteria, including a pathogen and several saprophytes isolated from apple.

This study extends the range of uses of PCR for detection of *E. amylovora* and defines the limits of sensitivity and specificity of the various techniques. Single-round PCR was simple to perform and required less than 1 day to complete, but it was less sensitive than the other methods (Fig. 1) and less specific than nested PCR. It is ideally suited for confirming bacterial colonies as *E. amylovora* and testing symptomatic plant tissue (3). We improved the sensitivity of detection of *E. amylovora* in apple tissue, a necessary step if this method is to be routinely applied in diagnostics.

Nested PCR was the most sensitive and specific of the detection assays and required about 1.5 days to complete. However, the risk of cross-contamination among samples and contamination from impure reaction mixture components is high and may preclude the routine application of nested PCR for diagnostic purposes. Including several negative controls interspersed among sample tubes would help assure that positive results were valid but also would entail added expense and effort. A "one-tube" nested PCR (20), in which first- and second-round primers are added simultaneously to a single reaction tube, might minimize the risk of contamination and save time and PCR reagents. If conducted properly, an extremely sensitive detection method such as nested PCR would prove valuable for indexing asymptomatic propagation material in an effort to prevent long-distance dissemination of *E. amylovora*.

PCR-dot-blot hybridization required 2 days to complete, and more handling and manipulation of samples than the other methods. Since high populations of bacteria other than *E. amylovora* sometimes resulted in false positives, it would be necessary to check for nonspecific products by gel electrophoresis before proceeding to dot-blot hybridization. Colorimetric detection, which is quicker and less expensive than chemiluminescent detection, was not reliable in the dot-blot assay because PCR products from plant samples stained membranes, thereby interfering with signal detection. Despite these drawbacks, PCR-dot-blot hybridization was more sensitive than single-round PCR (Table 2; Fig. 1), less prone to false positives than nested PCR, and would be convenient for screening large numbers of samples.

The reverse-blot method could be performed in less than 1 day because detection strips could be premade and stored indefinitely. Also, the method was amenable to colorimetric detection, and signals were often visible after only 5 min in color development solution. Moreover, the method did not require electrophoresis equipment. These features favor the development of the reverse-blot method into a commercial detection kit. Reverse-blot hybridization lacks the sensitivity, specificity, and ease of use with large numbers of samples required for screening propagation material

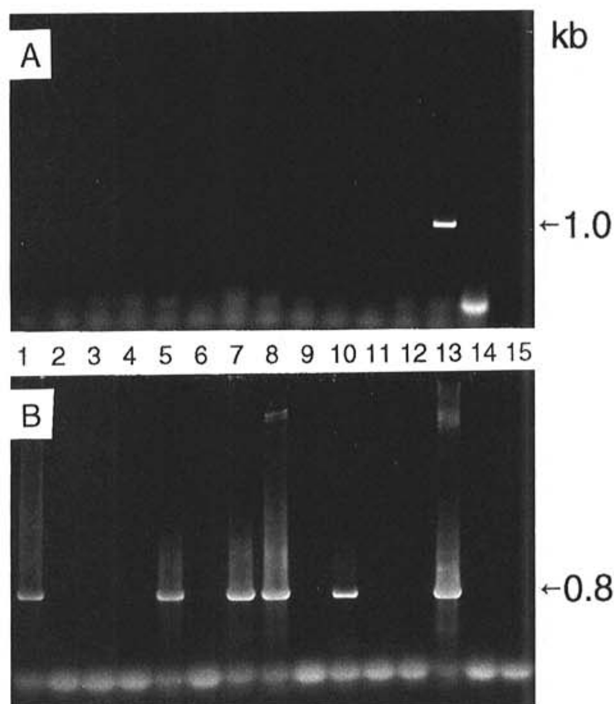

Fig. 3. Comparison of assays for detection of *Erwinia amylovora* in symptomless calyces of apple from an orchard with fire blight at Michigan State University, East Lansing. **A,** First-round polymerase chain reaction (PCR) products amplified from primers A and B. **B,** Nested PCR products of DNA from **A** amplified with internal primers AJ75 and AJ76. Lanes 1–12, individual calyces macerated in water; lane 13, macerated calyx tissue spiked with 5×10^5 colony forming units of *E. amylovora*; lane 14, macerated calyx tissue previously determined to be free of *E. amylovora*; and lane 15, **A,** empty lane; **B,** water negative control for nested PCR. PCR products were separated by electrophoresis through 1% agarose gels.

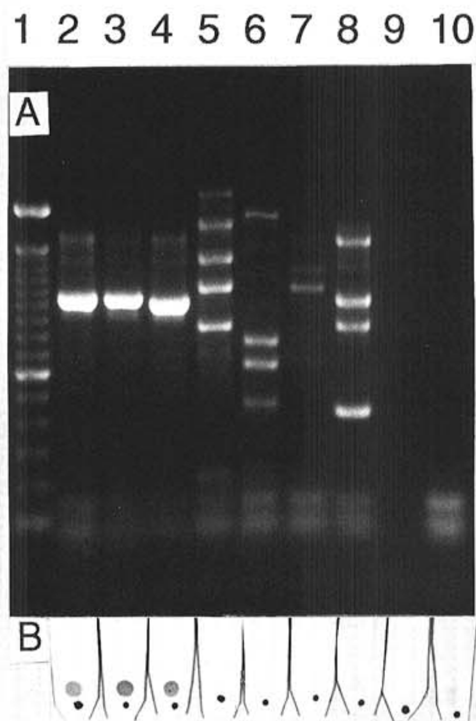

Fig. 4. Specificity of assays for *Erwinia amylovora*. **A,** Polymerase chain reaction (PCR) products of 10^5 colony forming units of various bacteria amplified from primers A and B. **B,** Reverse-blot hybridization. Digoxigenin-11-UTP was incorporated into PCR products amplified from primers A and B; labeled products were hybridized to probe N844, which had been immobilized on strips of nylon membrane; the small dots near the tips of the detection strips were applied as reference points for spotting N844 DNA. Lane 1, 100-bp ladder; lanes 2–4, *E. amylovora* strains CAll, BCN20, and EL01, respectively; lane 5, *E. herbicola* strain BC9; lane 6, *Pseudomonas syringae* pv. *papulans* strain Psp36; lane 7, unidentified bacterium isolated from an apple shoot; lane 8, *Escherichia coli* strain JM109; lane 9, PCR buffer without amplification; and lane 10, water control. PCR products were separated by electrophoresis through a 1% agarose gel.

for low populations of *E. amylovora*. However, the method would be quick and simple for testing colonies of putative isolates of *E. amylovora*, active fire blight infections, and possibly epiphytic *E. amylovora* on blossoms. Simultaneous detection of several pathogens might be possible by spotting different pathogen-specific probes onto detection strips and including corresponding pathogen-specific primers in a multiplex PCR.

Detection of dead cells might be problematic when screening shipments of fruit, budwood, or nursery stock since only viable bacteria would pose a threat of introducing fire blight. Culture enrichment prior to PCR was used to detect viable *Pseudomonas syringae* pv. *phaseolicola* in bean seeds (25) and *Salmonella* spp. in oysters (1). DNA from live cells might be selectively amplified from an mRNA template by reverse transcription PCR, assuming mRNA is intact and relatively abundant in live cells but degrades quickly after cell death. Live and dead cysts of *Giardia* were differentiated by comparing the amounts of PCR product amplified from an mRNA target (14). A molecular method to distinguish viable but nonculturable cells from dead cells of *E. amylovora* would be desirable, especially for monitoring the pathogen on mature fruit, because the ratio of dead to viable cells might be high at harvest time.

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