Chemiluminescent and Colorimetric Detection of *Erwinia amylovora* by Immunoenzymatic Determination of PCR Amplicons from Plasmid pEA29

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ABSTRACT

Merighi, M., Sandrini, A., Landini, S., Ghini, S., Girotti, S., Malaguti, S., and Bazzi, C. 2000. Chemiluminescent and colorimetric detection of *Erwinia amylovora* by immunoenzymatic determination of PCR amplicons from plasmid pEA29. Plant Dis. 84:49-54.

A molecular diagnostic technique (polymerase chain reaction enzyme-linked immunosorbent assay [PCR-ELISA]) for detection of *Erwinia amylovora* was developed. The protocol is based on the immunoenzymatic determination of PCR products. For in vitro amplification, we used previously published primers able to detect the cryptic plasmid pEA29, which is ubiquitous in *E. amylovora*. Amplicons were labeled with 11-digoxigenin (DIG)-dUTP during the amplification reaction, captured by hybridization to a biotinylated oligonucleotide in streptavidin-coated ELISA microplates, and then detected with anti-DIG-Fab'-peroxidase conjugated antibodies. The specificity of the assay was verified using *E. amylovora* strains from different host plants and geographical origins in addition to other plant-associated bacteria (either phytopathogenic or saprophytic) belonging to the genera *Erwinia*, *Pseudomonas*, and *Agrobacterium*. In detection threshold experiments with pure cultures, as few as 30 and 3 CFU/reaction tube were detected when the ABTS (colorimetric) and ECL (chemiluminescent) detection assays, respectively, were used. PCR-ELISA coupled with chemiluminescent detection was able to detect as few as 4×10^2 CFU/g of artificially infested pear twigs. The assay was further shown to be suitable for detection of *E. amylovora* in naturally infected plant organs, and the results were compared to those obtained using standard PCR assays with electrophoretic separation of amplicons.

The bacterium *Erwinia amylovora* is the causal agent of fire blight of apple (*Malus domestica*), pear (*Pyrus communis*), and many other rosaceous plants (27). Fire blight is the most destructive disease of cultivated fruit trees and of great economic importance worldwide. Available detection and identification methods include: (i) isolation of the pathogen on the semi-selective media of Miller and Schroth (MS) (23), Crosse and Goodman (CG) agar (4), crystal violet-cycloheximide-thallium nitrate (CCT) agar (14), and minimal medium 2-copper sulfate (MM2Cu) agar (2); (ii) pathogenicity tests (15); (iii) immunofluorescent microscopy (22) and double-antibody sandwich indirect enzyme-linked immunosorbent assay (DASI-ELISA; 8); (iv) analysis by gas chromatography of fatty acid methyl esters (GC-FAME) (28); and (v) molecular methods using both colony hybridization (5) and polymerase chain reaction (PCR), alone or in combination with DNA hybridization (1,3,9,21).

PCR diagnostic assays for *E. amylovora* are based mostly on the amplification of a

1-kilobase (kb) fragment (3) using primers from the plasmid pEA29, which is ubiquitous in all strains of this species. The use of plasmid DNA as a target for amplification allows a more sensitive detection of the pathogen as compared with chromosome-based regions (1). One drawback to this approach, however, is the detection of non-target bacteria associated with rosaceous plant tissues (3,21,30). False positive signals can be resolved by restriction endonuclease digestion of the amplification products (30) or by nested PCR (21). However, these assays are either time-consuming or prone to "carryover" contaminations by first-round PCR reactions. The detection of amplification products by dot-blot hybridization was shown to significantly enhance PCR sensitivity, thereby nullifying the carry-over problem, but was still slow and laborious. Moreover, in some cases, false positive signals were observed (21).

Alternative methods of detection of PCR products rely on the use of ELISA to detect labels incorporated in the amplicons (labeled primers or deoxynucleotides), with or without an intermediate DNA hybridization step using biotinylated or fluorescent probes (13,16,18,20,26). Once PCR reactions have been optimized or a specific oligonucleotide probe has been designed, these systems enable automated processing by ELISA microtiter plate readers, thereby streamlining the process. The use of PCR-

ELISA protocols has been successfully applied in human medicine (6), food microbiology (10), and, to a lesser extent, in plant virology (24) and bacteriology (11,12).

To our knowledge, these alternative approaches have not yet been applied to the molecular detection of *E. amylovora*. Given the economic importance and ramifications of fire blight, along with its aggressiveness and wide host range, more rapid and user-friendly detection methods are required to control commercial nursery stocks and plant propagation materials and to speed-up field surveys. The use of such PCR and ELISA combined techniques could have a significant impact on the management of this pathogen by providing epidemiologists and state extension pathologists with a rapid and selective detection procedure.

Having this in mind, an automated method was developed to enhance our ability to quickly detect *E. amylovora* in multiple samples. Specifically, we explored the immunodetection of digoxigenin (DIG)-labeled amplicons without gel electrophoresis analysis (Fig. 1). Previously described primers from the cryptic plasmid pEA29 (3) and 11-DIG-dUTP were used for in vitro DNA amplification and labeling during the reaction, respectively. An oligonucleotide hybridization probe (oligoprobe) was designed using published sequence information (21). The 5' biotinylated probe hybridized to an internal region of the resulting 1-kb amplicon, allowing the specific hybridization-capture of *E. amylovora* amplicons on streptavidin-coated microtiter plates. Anti-DIG-Fab' peroxidase conjugate antibodies were used to detect captured PCR products. The colorimetric PCR-ELISA protocol from Roche Molecular Biochemicals (Indianapolis, IN) was initially used for detection of amplicons. Subsequently, buffers were modified according to published work (6) and the use of a chemiluminescent substrate was introduced. This was compared with a commercially available colorimetric detection system and agarose gel electrophoresis. In the present study, the sensitivity and specificity of the proposed molecular technique was determined using both pure cultures and DNA extracted from experimentally contaminated plant material and compared. Finally, the specificity and sensitivity of the analytical technique were

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Accepted for publication 16 September 1999.

verified using naturally infected and asymptomatic field samples.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Bacteria were grown overnight in Luria-Bertani (LB) broth (25), on King's medium B (KB agar) (17), or on nutrient sucrose agar (NSA; 15) medium for 48 h at 27°C.

Plant materials. A total of 116 samples from symptomatic plant organs (blossom, fruits, leaves, shoots, and twigs with cortical cankers) were collected from 19 blighted pear trees (cvs. Conference, Abbé Fétel, and Kaiser) in five orchards in the district of Bologna (Italy). Naturally in-

fectected apple samples were not available. Asymptomatic 1-year-old pear twigs were also collected and served either as negative controls or for the in planta detection-threshold experiments. Samples were stored at 4°C in plastic bags.

Experimental contamination of plant material. For detection-threshold experiments, pear twig cuttings (1 g each) were used. Samples were infested by adding aliquots of 10-fold serial dilutions of aqueous bacterial suspensions of *E. amylovora* strain OMP-BO1077.7, then dried under vacuum for 10 min. Dried samples were assayed the same day.

Extraction of bacteria from plant samples. Single tissue samples (approximately 500 mg) were ground with a mortar

and pestle in 4 ml of antioxidant buffer (phosphate-buffered saline [PBS], pH 7.2, with 2% polyvinyl pyrrolidone-10, 1% mannitol, 10 mM ascorbic acid, and 10 mM reduced glutathione, sterilized by filtration; 8). Homogenates were allowed to settle for 30 min prior to filtration through Whatman No. 1 paper. Volumes (2 ml) were centrifuged in Eppendorf tubes at $16,000 \times g$ for 5 min and the pellet was stored at -20°C.

Direct isolation and identification of bacteria. Paper-filtered plant extracts (30 µl) were also used for direct isolation on KB agar. Non-fluorescent colonies were checked for levan production on NSA and for the presence of cytochrome-c activity (15). Levan-producing, oxidase-negative strains were infiltrated into 6-week-old tobacco leaves and inoculated into young pear slices (15). Strains producing hypersensitive reaction plus ooze and necrosis on pear were presumptively identified as *E. amylovora*.

DNA purification from plant extract pellets. Each plant extract pellet was resuspended in 700 µl of 10 mM Tris-HCl, pH 8.0, and total genomic DNA was extracted according to the cetyltrimethylammonium bromide (CTAB) miniprep procedure of Wilson (29), modified to include a second chloroform:isoamyl alcohol (24:1) extraction. DNA was precipitated with 0.7 volumes of isopropanol and redissolved in 100 µl of 10 mM Tris-HCl, 1 mM Na₂-EDTA, pH 8.0 (TE_{10/1}).

PCR amplification. DNA amplification was carried out using a 20-µl reaction volume containing 5 µl of whole-cell bacterial suspension, genomic DNA solution (purified from overnight bacterial cultures as described above and diluted at 10 ng/µl as determined by A₂₆₀ spectrophotometry), or 5 µl of water (no-DNA negative PCR controls); 50 mM KCl; 10 mM Tris-HCl pH 9.0; 2 mM MgCl₂; 0.1% Triton X-100, 0.2 mM each dATP, dGTP, dCTP, and dTTP; 20 pmol each of primers A and B (3); and 0.8 U of *Taq* DNA polymerase (Promega, Madison, WI). Amplification was performed in a thermal cycler (GeneAmp PCR System 2400, Perkin Elmer, Norwalk, CT) programmed for one cycle of 5 min at 94°C, followed by 38 cycles of 1 min at 94°C, 30 s at 52°C, and 1 min at 72°C, with a final extension of 10 min at 72°C.

Gel electrophoresis of PCR products. PCR products were analyzed by electrophoresing 10 µl of the reaction mixture in a Tris-acetate-EDTA agarose gel (1.5%; 25), staining with ethidium bromide (1 µg/ml), and visualizing with UV light.

Oligonucleotide hybridization probe design. The sequence of the 1-kb fragment amplified by primers A and B from *E. amylovora* CA11 (21) was available from GenBank (accession number U19254). The Primer Designer program (v. 3.0; Scientific & Educational Software, Stateline, PA)

Fig. 1. Scheme of the polymerase chain reaction enzyme-linked immunosorbent assay (PCR-ELISA) protocol. DNA extracted from (1) infected plant samples or (2) aqueous suspensions of *Erwinia amylovora* cells were used as a template in PCR and amplicons were labeled with (3) 11-digoxigenin-dUTP during PCR. (4) Labeled amplicons were denatured and hybridized to an internal oligonucleotide biotinylated in 5'. Molecular hybrids were (5) captured in streptavidin-coated ELISA microtiter wells and (6) detected by an immunoenzymatic assay using colorimetric or chemiluminescent substrates.

was used to design oligonucleotide probes. Capture oligoprobes were then synthesized as 5'-biotinylated oligonucleotides at Gibco-Life Technologies (Edinburgh, UK).

PCR-ELISA assay. For nonradioactive labeling during PCR, we used the same primers and conditions described above except that 11-DIG-dUTP was used together with dTTP at a ratio of 1:19 (Roche Molecular Biochemicals) and combined molar concentration of 0.2 mM. A 10- μ l volume of amplification product was added to 10- μ l of denaturation solution (100 mM NaOH, 0.1% Tween 20; 6) and incubated for 10 min at room temperature. Subsequently, 200- μ l aliquots of hybridization solution (300 mM NaCl, 100 mM Tris-HCl, pH 6.5, 0.1% Tween 20; 6) containing different concentrations of 5'-biotinylated oligoprobes (1, 10, and 50 pmol ml⁻¹) were added to each denatured sample. Aliquots of 220 μ l were loaded on streptavidin-coated ELISA microtiter plates and incubated for 3 h at room temperature, 37°C, and 55°C in a thermal-controlled water bath under moderate agitation (60 rpm). For colorimetric detection (Col-PCR-ELISA), transparent microtiter plates were used; whereas, for chemiluminescent detection (Chem-PCR-ELISA), black plates were used. After the hybridization-capture step, wells were washed four times with TBST (100 mM NaCl, 100 mM Tris-HCl, pH 7.5, 0.1% Tween 20; 6) and incubated 1 h at room temperature with 200 μ l of anti-DIG-peroxidase conjugate antibodies (Roche Molecular Biochemicals) in TBST at the final concentration of 1 U ml⁻¹. After incubation, microwells were washed three to five times with TBST. For colorimetric detection in ELISA microtiter plates, 200 μ l of the enzymatic substrate 2,2'-azine-di-3-ethyl-benzthiazoline (ABTS; Roche Molecular Biochemicals) were added to each well. Plates were incubated for 30 min at 37°C in the dark and photometrically scanned ($A_{405\text{ nm}}$) up to 1 h after the first reading. For chemiluminescent detection, 100- μ l volumes of the enzymatic substrate ECL (Amersham Life Science, cat. no. 2106L) were mixed with 100 μ l of manufacturer's buffer in ELISA microtiter wells, and immediately read in luminometry (light emission in photons/sec) up to 30 min after the start of reactions. In both cases, plate analyses were performed using a Victor 1420 multilabel reader (EG&G Wallac, Gaithersburg, MD). The cut-off limit for PCR-ELISA detection was defined as two times the average reading ($A_{405\text{ nm}}$ or light emission) for negative PCR control samples (no DNA for experiments with pure cultures, and DNA extracted from non-contaminated control samples for treatments with experimentally infested pear twigs).

RESULTS

Oligonucleotide hybridization probe design. Several 20- and 50-mer oligo-

probes were designed between positions 310 and 520 of the 1-kb amplicon (GenBank accession number U19254). After preliminary tests with several oligoprobes of different length, the 50-mer oligonucleotide designated MM7 (5'-CAG-GAACGACATCCGGCTTCTGAAATACGCCTGA-CCACCTCAACTGATA-3') was chosen for PCR-ELISA experiments. Shorter probes (20 mer) always showed low signal intensity for positive samples and high background with negative controls (*not shown*).

Optimization of PCR-ELISA. Several combinations of probe concentration, hybridization temperature, capture time, and post-hybridization washes were tested to

determine optimal reaction conditions ensuring the highest positive signal with the lowest background (*data not shown*). Whole-cell suspensions of *E. amylovora* OMP-BO1077.7 (about 10⁵ CFU/reaction tube) were used as a template for DNA amplification steps. Optimal conditions were set at 10 pmol of biotinylated probe/1 ml of hybridization buffer, with a hybridization temperature of 55°C for 3 h and four post-hybridization washes. Time-course experiments (*data not shown*) revealed that the colorimetric signal reached saturation after 60 min at 37°C. In contrast, the chemiluminescent assay could be read immediately. The same Col-PCR-ELISA reaction parame-

Table 1. Bacterial strains analyzed by polymerase chain reaction enzyme-linked immunosorbent assay

Strain ^a	Host	Origin	Source
<i>Erwinia amylovora</i>			
OMP-BO1077.7	Pear	Italy, 1994	A. Calzolari
OMP-BO1204.1	<i>Pyracantha</i> spp.	Italy, 1994	A. Calzolari
OMP-BO1178.1	<i>Sorbus</i> spp.	Italy, 1994	A. Calzolari
OMP-BO1160.2	Hawthorn	Italy, 1994	A. Calzolari
OMP-BO786.1	Pear	Italy, 1991	R. D'Anna
109BA	Pear	Italy, 1990	A. Cariddi
11LE	Pear	Italy, 1990	A. Cariddi
BPIC 847	Pear	Greece, 1984	P. G. Psallidas
BPIC 913	Pear	Greece, 1985	P. G. Psallidas
BPIC 928	Pear	Greece, 1986	P. G. Psallidas
Ea 1/79	<i>Cotoneaster</i> spp.	Germany, 1979	(5)
CFPB1367	Hawthorn	France, 1978	J. P. Paulin
CFPB2543	Apple	France, 1992	P. Lecomte
295/93	<i>Cotoneaster</i> spp.	Austria, 1993	M. Keck
674/94	<i>Cotoneaster</i> spp.	Austria, 1994	M. Keck
331.93	Pear	Switzerland, 1993	T. Hasler
345.90	<i>Cotoneaster</i> spp.	Switzerland, 1990	T. Hasler
508.94	Hawthorn	Switzerland, 1990	T. Hasler
FB2a	Crab apple	Spain, 1995	M. Lopez
BPIC 1631	Hawthorn	Czech Rep., 1989	V. Kudela
115.22	Crab apple	Bulgaria, 1989	S. Bobev
202.1	Crab apple	Bulgaria, 1989	S. Bobev
IPV-BO 2906/95	Pear	Albania, 1995	U. Mazzucchi
CA3R	Apple	United States	(21)
NZS4	Pomaceous plants	New Zealand	(21)
OR6	Pear	United States	(21)
IL6	<i>Rubus</i> spp.	United States	(21)
8865nrRI	<i>Rubus</i> spp.	United States	(21)
Ea 8/96	<i>Rubus</i> spp.	Canada, 1996	K. Geider
<i>P. agglomerans</i>			
C9	Apple	United States	(21)
<i>Pseudomonas syringae</i> pv. <i>syringae</i>			
OMP-BO 443	Pear	Italy, 1993	A. Calzolari
IPV-BO 1326	Pear	Italy, 1980	C. Bazzi
IPV-BO 2204	Pear	Italy, 1986	U. Mazzucchi
<i>P. syringae</i> pv. <i>papulans</i>			
NCPBP 3262	Apple	Italy, 1981	C. Bazzi
<i>P. fluorescens</i>			
OMP-BO 90.1	Garlic	Italy, 1990	A. Calzolari
TL-3	Potato	United States, 1980	T. J. Burr
<i>P. putida</i>			
TS034	Sugar beet	United States, 1978	T. V. Suslow
<i>E. carotovora</i> ssp. <i>carotovora</i>			
NCPBP 312	Potato	Denmark, 1952	E. Hellmers
<i>Agrobacterium tumefaciens</i> biovar 1			
IPV-BO 2028	Daisy	Italy, 1984	C. Bazzi
<i>Escherichia coli</i>			
JM109			Promega, USA

^a IPV-BO = Istituto di Patologia Vegetale, Università di Bologna, Italy; OMP-BO = Regional Phytosanitary Service-Bologna (former Osservatorio per le Malattie delle Pianta), Italy; BPIC = Benaki Phytopathological Institute Collection, Greece; CFPB = Collection Française des Bactéries Phytopathogènes, France; and NCPBP = National Collection of Plant Pathogenic Bacteria, UK.

ters were optimal also for Chem-PCR-ELISA (data not shown).

Specificity of PCR-ELISA. The 50-mer oligoprobe MM7 was tested for its ability to hybridize specifically with the 1-kb amplicon from plasmid pEA29. Both whole-cell bacterial suspensions (about 10^5 CFU/reaction tube) and genomic DNA (about 50 ng) were used for PCR amplifications. All of the *E. amylovora* strains used in this study gave a clear positive hybridization signal when tested with PCR-ELISA (average $A_{405} = 1.68 \pm 0.22$ for Col-PCR-ELISA). Other bacteria often found associated with apple and pear, either as saprophytes or as plant pathogens, (e.g. *Pantoea agglomerans*, *Pseudomonas putida*, *P. fluorescens*, and *P. syringae* pathovars) and strains belonging to other *Erwinia* spp. and related genera (namely *Escherichia coli*) gave hybridization signals indistinguishable from the negative PCR control (no DNA). Experiments were done three times, using both bacterial genomic DNA and whole cells, with similar results.

Sensitivity of PCR-ELISA and comparison with standard PCR and electrophoresis detection. Detection-threshold experiments were performed using whole cells of *E. amylovora* OMP-BO1077.7 and genomic DNA extracted from experimentally infested pear twig cuttings. Experiments were done three times with two replicates per sample. In general, the detection threshold using the colorimetric protocol was at least 10 times higher than the threshold using the chemiluminescent detection. Col-PCR-ELISA showed an unambiguous positive-result signal down to 3×10^2 CFU/reaction tube, and a near-to-cut-off signal with 30 CFU/reaction (Table 2); whereas, using DNA from infected twigs gave a detection threshold of 4×10^3 CFU/g of plant tissue (Fig. 2). On the other hand, Chem-PCR-ELISA was able to unambiguously detect as few as 3 CFU/reaction tube (Table 2) and 4×10^2 CFU/g of plant tissue (Fig. 3). Standard PCR using whole cells of *E. amylovora* in pure culture and infested plant tissue followed by gel electrophoresis analysis gave a sensitivity threshold of 3×10^2 CFU/reaction tube and 4×10^4 CFU/g of

plant tissue, respectively (Table 2). Absorbance values were less reproducible from experiment to experiment in comparison to light emission readings in experiments with pure cultures: relative standard error (SE divided the square root of the number of readings per cell dilution) ranged from 2.4 to 33% for Col-PCR-ELISA and from 2.8 to 19% for Chem-PCR-ELISA. In general, higher cell concentration showed lower variability, as expected.

Comparison of PCR-ELISA, PCR, and direct isolation using naturally infected plant material. A total of 116 naturally infected pear fire blight samples were extracted as described above. Samples were assayed by Chem-PCR-ELISA and PCR, after total DNA purification in two independent experiments. Aliquots were also used for direct isolation and microbial identification by classic techniques (Table 3). As expected, PCR was more sensitive than direct isolation in most cases, especially using infected tissues where the bacterial population reached lower concentrations, as in old cankers. On the other hand, Chem-PCR-ELISA showed a higher sensitivity compared to PCR, with overall agreement between the two approaches: samples found positive with PCR were always positive by Chem-PCR-ELISA, but 93% of samples negative by PCR were

found positive using Chem-PCR-ELISA. As a control for specificity, healthy-appearing samples (a total of 15, including different sources of tissue) were always found negative using the three methods.

DISCUSSION

The PCR-ELISA assay proposed in this study (Fig. 1) is an improvement, in terms of sensitivity and through-put, over a PCR-based detection method currently available for the identification of *E. amylovora* (3). PCR with primers A and B, which amplify a 1-kb fragment from the cryptic plasmid pEA29, was chosen for this PCR assay because it allows lower detection thresholds compared to chromosomal targets (1). The new 50-mer biotinylated oligoprobe designed from the sequence of the 1-kb PCR fragment has allowed the specific detection of bacterial DNA by combined capture-hybridization and immunoenzymatic assay instead of using analytical gel electrophoresis. The added use of this oligoprobe eliminates the occasional problems with multiple products and poor specificity reported elsewhere when using PCR alone (3,21,30). Other commercially available immunocapture-PCR methods not including a DNA hybridization step were not considered because of the risk of false positives if not properly optimized.

Fig. 2. Colorimetric polymerase chain reaction enzyme-linked immunosorbent assay: detection threshold determination using experimentally infested pear tissues. Concentration of bacteria in plant tissues are expressed as logarithms. Bar refers to confidence intervals ($P = 0.95$).

Fig. 3. Chemiluminescent polymerase chain reaction enzyme-linked immunosorbent assay: detection threshold determination using experimentally infested pear tissues. Concentration of bacteria in plant tissues are expressed as logarithms. Light emission expressed in photon per second is showed in logarithmic scale. Bar refers to confidence intervals ($P = 0.95$).

Table 2. Comparison of the sensitivity of detection using polymerase chain reaction enzyme-linked immunosorbent assay (PCR-ELISA) and standard PCR on whole-cell suspensions of *Erwinia amylovora*

Detection method	CFU/reaction tube ^a						
	0 ^b	3	3×10	3×10^2	3×10^3	3×10^4	3×10^5
Chem-PCR-ELISA ^c	90 $\pm$ 3	180 $\pm$ 9	290 $\pm$ 9	1,680 $\pm$ 60	13,080 $\pm$ 719	36,960 $\pm$ 5,500	119,450 $\pm$ 5,370
Col-PCR-ELISA ^d	0.17 $\pm$ 0.02	0.22 $\pm$ 0.02	0.36 $\pm$ 0.05	1.02 $\pm$ 0.11	1.38 $\pm$ 0.17	1.49 $\pm$ 0.23	1.60 $\pm$ 0.27
Chem-PCR-ELISA results	—	+	+	+	+	+	+
Col-PCR-ELISA results	—	—	+/- ^e	+	+	+	+
PCR	—	—	—	+	+	+	+

^a Average value of three independent experiments $\pm$ standard deviation.

^b Negative PCR control (no DNA).

^c Chemiluminescent PCR-ELISA; light emission in photons per second.

^d Colorimetric PCR-ELISA; absorbance at 405 nm.

^e Results near to the cut-off, which was two times the average reading ($A_{405 \text{ nm}}$ or light emission) for the negative PCR control.

All of the *E. amylovora* strains tested in this study showed a strong positive signal after the capture-hybridization and detection steps, including those strains (e.g., BU202.1 and strains from Austria) that were reported to have length polymorphisms of the 1-kb amplicon (19; M. Merighi, unpublished). Temperatures above 50°C were essential in order to obtain a low background and a high specificity of the assay (data not shown). Shorter probes would have allowed lower hybridization temperatures, but the 20-mer oligoprobes tested gave low signal-to-noise ratio (data not shown). Preliminary experiments were conducted using the reagents provided by the manufacturer's colorimetric kit (Roche Molecular Biochemicals) but, subsequently, we modified the hybridization, immunological buffers, and detection substrate as described above, with comparable results. Using chemiluminescent substrates, the detection threshold was from 10 to 100 times lower than those found for standard PCR both in this (Table 2) and previous works (1,21), whereas colorimetric detection protocol produced results at best comparable with PCR (i.e., from 30 to 3×10^2 CFU/reaction tube; Table 2). The efficiency of the capture hybridization and ELISA reactions, along with the greater sensitivity of chemiluminescent substrates compared to chromogenic tracers (7,12), may account for the lower detection threshold of Chem-PCR-ELISA compared to PCR. Results of experiments with artificially infested and naturally infected plant materials (Table 3) were consistent with those using pure cultures (i.e., higher specificity and sensitivity of Chem-PCR-ELISA compared to PCR), confirming the suitability of the protocol for in planta detection. To prepare DNA from infested and infected plant tissues, we applied a common protocol, including CTAB and organic solvents (29), reportedly able to overcome problems of inhibition of the PCR reaction when using crude extracts. We did not attempt to improve the sensitivity by modifying the DNA extrac-

tion procedure, but it is obvious that space exists to decrease the threshold of the assay in term of CFU per mass of plant material (e.g., using larger samples and differential centrifugation, extraction of bacteria by sonication, DNA purification by anion-exchange chromatographic methods, and so on). Finally, the proposed diagnostic test could be completed in less than 6 h and was suitable for analysis of 96 samples in the same microplate; in addition, the use of an ELISA multilabel reader enabled automation of the detection step.

Our chemiluminescent protocol could be of great value for the screening of large numbers of samples for plant certification and epidemiology studies. On the other hand, the cost of the luminometer reader could represent a major limitation for smaller laboratories, which could opt to use the colorimetric method (as sensitive as PCR in the worst case), increasing the laboratory through-put compared to gel electrophoresis analysis. Further modifications to the assay to carry out the amplification steps in ELISA microplates could completely automate the analysis. Moreover, the PCR-ELISA assay could be easily modified as a competitive PCR able to better distinguish among negative and false-negative results and to quantify the total amount of target DNA, as has been done for human pathogens (6,10). Work is in progress in our laboratories to extend the assay in this direction.

ACKNOWLEDGMENTS

We thank D. L. Coplin, M. Boehm, and D. Y. Han for critical reading of the manuscript; and the researchers reported in Table 1 for providing bacterial strains.

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Table 3. Assays on symptomatic pear organs and percentage of positive samples using direct isolation, polymerase chain reaction (PCR), and chemiluminescent PCR enzyme-linked immunosorbent assay (Chem-PCR-ELISA)^a

Plant organs ^b	No. of samples	Incidence (%) of positive detection of <i>Erwinia amylovora</i>		
		Direct isolation	PCR-AGE ^c	Chem-PCR-ELISA ^d
Leaves	22	61	81	91
Cankers	30	63	63	93
Shoots	31	52	56	80
Flowers	27	67	74	96
Fruits	6	100	100	100

^a Assays on asymptomatic pear samples (for a total of 15) were always negative.

^b Samples of symptomatic naturally infected plant tissues from infected pear plants were extracted by grinding with a mortar and pestle. For direct isolation procedure, aliquots of extracts were plated on King's medium B agar and identified; for PCR-based methods, samples were centrifuged and DNA extracted.

^c PCR-AGE = standard PCR and detection of amplicon by agarose gel electrophoresis.

^d Chem-PCR-ELISA = PCR-ELISA with chemiluminescent detection of the amplicon-oligoprobe hybrids captured in ELISA wells.

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In the article "Chemiluminescent and colorimetric detection of *Erwinia amylovora* by immunoenzymatic determination of PCR amplicons from plasmid pEA29" by M. Merighi, A. Sandrini, S. Landini, S. Ghini, S. Girotti, S. Malaguti, and C. Bazzi, pages 49 to 54, on page 51, the first full sentence in the second column should read,

After preliminary tests with several oligoprobes of different length, the 50-mer oligonucleotide designated MM7 (5'-ACAGGAACGACATC-CGGCTTCTGAAATACGCCTGACCACCTCAACTGATA-3') was chosen for PCR-ELISA experiments.