Comparison of PCR, ELISA, and DNA Hybridization for the Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in Field-Grown Potatoes

S. A. Slack, J. L. Drennan, and **A. A. G. Westra,** Department of Plant Pathology, Cornell University, Ithaca, NY 14853; **N. C. Gudmestad**, Department of Plant Pathology; and **A. E. Oleson**, Department of Biochemistry, North Dakota State University, Fargo 58105

ABSTRACT

Slack, S. A., Drennan, J. L., Westra, A. A. G., Gudmestad, N. C., and Oleson, A. E. 1996. Comparison of PCR, ELISA, and DNA hybridization for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* in field-grown potatoes. Plant Dis. 80:519-524.

Clavibacter michiganensis subsp. *sepedonicus*, the causal agent of bacterial ring rot, was specifically detected in field-grown potatoes by means of the polymerase chain reaction (PCR). A 20-bp synthetic oligomer, derived from an inverted repeat region of the repeated sequence of the bacterial plasmid pCS1, was used as the PCR primer. In assays of pure bacterial cultures, this system gave positive reactions with all strains of *C. michiganensis* subsp. *sepedonicus* tested and had a sensitivity of <10 CFU per PCR reaction. With the exception of 4 out of 12 strains of *C. michiganensis* subsp. *insidiosus*, no other species of bacteria tested produced a specific PCR product. The ability of PCR to detect *C. michiganensis* subsp. *sepedonicus* in field material was evaluated by testing sections of potato stems that were grown in New York and North Dakota from seed pieces of potato cultivars BelRus and Russet Burbank inoculated with 0, 10^2, or 10^9 CFU *C. michiganensis* subsp. *sepedonicus* and destructively sampled at 35 and 90 days after planting (DAP). Parallel tests of these samples by enzyme-linked immunosorbent assay (ELISA) and DNA hybridization assay (DHA) were conducted for comparative purposes. Overall, 36.2, 35.8, and 29.1% of inoculated samples tested positive by PCR, ELISA, and DHA, respectively. Each assay was affected significantly by inoculum dose, cultivar, and sampling date ($P = 0.0001$), with detection approaching 100% for the combination of the following parameters: 90 DAP, susceptible cv. Russet Burbank, and 10^9 CFU inoculum level. None of the buffer-inoculated plants tested positive with either PCR or DHA, whereas ELISA results were highly dependent upon the positive-negative threshold used.

Despite more than 50 years of a zero tolerance limit on bacterial ring rot in seed potatoes, this disease remains the primary cause of rejection of seed potato lots for certification in North America (9). Because the primary method of enforcing the zero tolerance limit is through visual inspection of seed potato lots, factors that interfere with the ability of inspectors to detect the disease, e.g., symptomless infection, environmental conditions unfavorable for symptom development, variability in cultivar response to the causal organism *Clavibacter michiganensis* subsp. *sepedonicus*, and interference from other diseases or the natural senescence of the host directly affect the efficacy of this control method (9,29). The difficulty of diagnosing bacterial ring rot based on symptom expression alone has led to the development and use of several assays for this disease, including the gram stain (14), eggplant bioassay (3), and several serological tests, e.g., latex agglutination (27), immunofluorescence (5,6,26), and enzyme-linked immunosorbent assay (ELISA) (6). While these assays have proven useful, their utility has been limited by labor and space constraints, sensitivity and/or specificity (5,19), and the time required to perform the assay.

Recently, interest has been shown in using DNA hybridization assays (DHAs) to detect and diagnose bacterial ring rot (13,16,20,24). Previous investigations by our laboratory indicate that this assay is highly specific for *C. michiganensis* subsp. *sepedonicus*, but that assay sensitivity is a limiting factor (12,30). One possible method of circumventing the problem of low assay sensitivity is to use the polymerase chain reaction (PCR) (17,24,25). This study details the results of our investigation into using PCR to detect and diagnose bacterial ring rot in crude extracts of field-grown potatoes and presents data that compare PCR with ELISA and DHA. A preliminary report of this data has been published (11).

Corresponding author: S. A. Slack
E-mail: ss18@cornell.edu

Accepted for publication 26 January 1996.

Publication no. D-1996-0226-04R

MATERIALS AND METHODS

Bacterial culture and inoculum preparation. *C. michiganensis* subsp. *sepedonicus* strain SS43 was maintained in lyophilized form in a 7% peptone and 7% sucrose solution and cultured on nutrient broth-yeast extract agar (NBY) (28) at ambient temperature (approximately 23°C). After 5 to 7 days of growth on NBY, bacteria were transferred to 1-liter flasks containing 500 ml of NBY broth and grown on a Labline 3520 orbital shaker (Labline Instruments, Melrose Park, IN) for 1 week at 150 rpm and ambient temperature. Bacteria were then harvested by centrifugation for 30 min at 15,000 × *g* at 4°C, resuspended in 0.05 M phosphate buffer pH 7.2 (PB), and adjusted to cell densities of 10^4 or 10^{11} CFU/ml in PB based on A_{600} being equivalent to 1.0×10^8 CFU/ml.

Experimental design and preparation. A factorial treatment design of inoculum dose and cultivar (3 × 2) was used and treatments were assigned to plots according to a randomized complete block design. Identical experiments, consisting of six blocks (15 plants per plot), were planted near Fargo, ND, and Ithaca, NY. Seed preparation and inoculation was performed at Ithaca. Seed to be planted at Fargo was shipped via overnight mail. Certified seed potatoes (cvs. BelRus and Russet Burbank) were removed from cold storage 1 week prior to cutting. Uniform seed pieces (approximately 40 g), each with a single eye, were then cut and placed in paper bags containing vermiculite dampened with a 3 g per liter Penncozeb solution and allowed to suberize for 2 to 3 days. Seed pieces were then wounded by inserting a pipet tip at a 45° angle on either side of the sprout and inoculated with 10 µl of bacterial suspension to give final inoculum doses of 10^2 or 10^9 CFU. Control plants were inoculated with 10 µl of sterile PB. After inoculation, the seed was returned to the paper bags described above and stored at ambient temperature until planting.

Sample preparation and testing. At 35 and 90 days after planting (DAP), five plants per plot were destructively sampled by collecting the main stem at or below the soil line. Samples collected in North Dakota were sent to New York via express mail for processing. Samples were washed under cold tap water, directly blotted onto nylon membranes as previously described (12), and then sealed in plastic bags and stored at −135°C. After a minimum of 24 h, samples were removed from the freezer, thawed slightly and again blotted onto nylon membranes. Nylon membranes were

then hybridized as previously described (12). Small (1.0 g) sections were then cut and ground in 2 ml of TE solution (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0) in preparation for testing by ELISA and PCR. These extracts were placed in microfuge tubes and stored at –80°C. Samples were prepared for ELISA by adding 100-ml aliquots of the TE extracts to 900 ml of extraction buffer (0.4% bovine serum albumin [BSA], 2% polyvinylpyrrolidone M_r 24,000 to 40,000, 0.13% Na_2HPO_4, 0.02% KH_2PO_4, 0.02% KCl, 0.05% Tween 20, pH 7.4) and the assay performed according to the protocol provided by the manufacturer (Agdia Inc., Elkhart, IN). The ELISA system used in this study is based upon a monoclonal antibody (1H3) for *C. michiganensis* subsp. *sepedonicus* described by De Boer et al. (7,10).

Polymerase chain reaction. A 20-bp synthetic oligomer, CSRS-C (5′-GGCCAT GACGTTGGTGACAC) (Operon Technologies, Inc., Alameda, CA), was used to amplify a 1.054-kb fragment of the repeated sequence of the bacterial plasmid pCS1. Two microliters of the original sample extract in TE was added to 198 µl of distilled water and mixed gently. Twenty microliters of the diluted extract was then added to 20 µl of freshly prepared lysis buffer (1.25% sodium dodecyl sulfate [SDS], 0.5 mM NaOH) and heated at 90°C for 15 min. Five microliters of the lysed cell suspension was then added to 45

Table 1. Reaction of bacteria tested by polymerase chain reaction (PCR) with primer CSRS-C

Organism	Number of strains tested	Reaction
Clavibacter michiganensis subsp. *sepedonicus*	52	+[a]
C. m. subsp. *insidiosus*	12	+/–[b]
C. m. subsp. *michiganensis*	2	–
C. m. subsp. *nebraskensis*	1	–
Curtobacterium flaccumfaciens subsp. *flaccumfaciens*	2	–
C. f. subsp. *poinsettiae*	2	–
Erwinia carotovora subsp. *atroseptica*	1	–[c]
Erwinia carotovora subsp. *carotovora*	2	–[c]
Pseudomonas spp.	1	–
Pseudomonas fluorescens	1	–
Pseudomonas solanacearum	2	–
Pseudomonas syringae pv. *glycineae*	1	–[c]
Pseudomonas syringae pv. *tomato*	1	–[c]
Rhodococcus facians	1	–
Saccharomyces cerevisiae	1	–
Xanthomonas campestris subsp. *translucens*	1	–[c]
Unidentified	13	–[c,d]

[a] Includes nonfluidal and sugar beet strains.
[b] Four of 12 strains tested yielded PCR product similar to that observed for *C. m.* subsp. *sepedonicus*.
[c] Includes strains that yielded nonspecific PCR product.
[d] Includes 8 strains shown by Crowley and De Boer (5) to react with polyclonal antisera for *C. m.* subsp. *sepedonicus*.

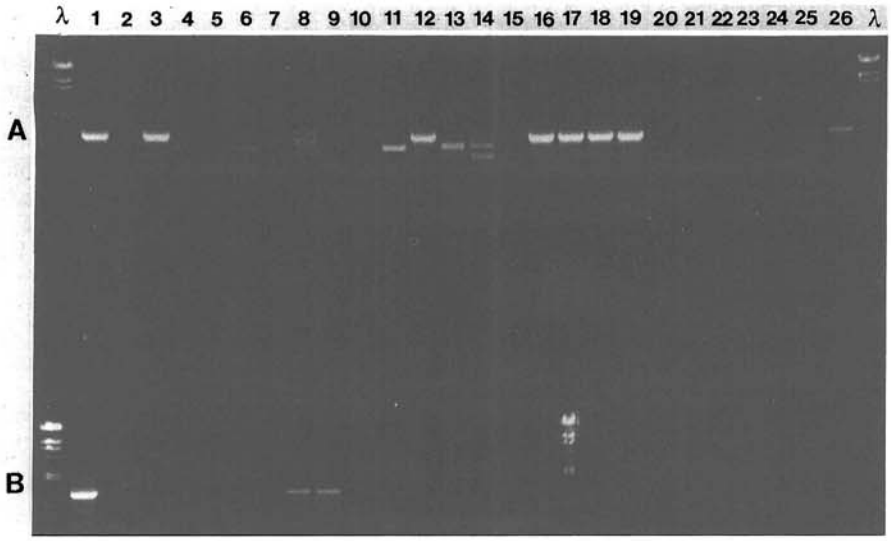

Fig. 1. Results of polymerase chain reaction (PCR) tests of *Clavibacter michiganensis* subsp. *sepedonicus* and other bacteria taken from pure culture using primer CSRS-C. PCR product was run on 0.6% agarose gels and stained with ethidium bromide. Row A: lane 1, *C. m.* subsp. *sepedonicus,* SS43 (positive control); lane 2, Tris-EDTA (TE) buffer (negative control); lane 3, *C. m.* subsp. *sepedonicus,* SS76; lanes 4 to 11, unidentified bacteria isolated from potato by De Boer (5); lane 12, *C. m.* subsp. *sepedonicus*, SS87; lane 13, *Erwinia carotovora.* subsp. *atroseptica,* SS88; lane 14, *E. c.* subsp. carotovora, SS91; lane 15, unidentified bacterium isolated from potato tubers, SS92; lanes 16 to 19, *C. m.* subsp. *sepedonicus* strains SS93-96; lanes 20 to 24, unidentified bacteria isolated from potato stems and tubers, SS98-102; lanes 25 to 26, *C. m.* subsp. *insidiosus* strains SS105-106. Row B: lane 1, *C. m.* subsp. *sepedonicus,* SS43 (positive control); lane 2, TE buffer (negative control); lanes 3 to 10, *C. m.* subsp. *insidiosus* strains SS107-114; lane 11, *Xanthomonas campestris* subsp. *transluciens*, SS115; lane 12, *Pseudomonas fluorescens*, SS116; lane 13, *P. solanacearum*, SS117; lane 14, *Saccharomyces cervisiae*, SS118; lane 15, *E. c.* subsp. *carotovora*, SS119; lane 16, *P. syringae* subsp. *glycineae*, SS120. Ladders (λ) are *Bam*HI-digested λ DNA.

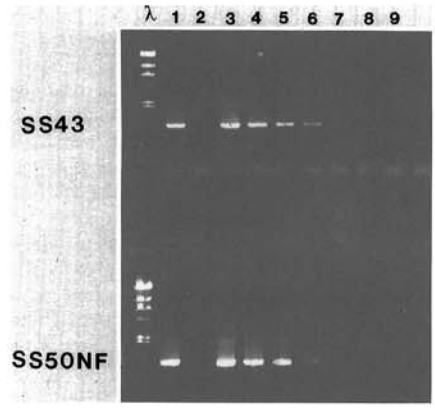

Fig. 2. Dilution series of *Clavibacter michiganensis* subsp. *sepedonicus* strains SS43 (fluidal) and SSNF50 (nonfluidal) tested by polymerase chain reaction (PCR) using CSRS-C primer. Positive (>10^{10} CFU/ml SS43) and negative (H_20) controls are shown in lanes 1 and 2, respectively. Lanes 3 to 9 represent serial, 10-fold dilutions of bacterial suspensions in H_20. Initial cell concentrations of lane 3 are 2.83×10^3 and 4.08×10^3 CFU per PCR reaction for SS43 and SSNF50, respectively. Ladders (λ) are *Bam*HI-digested λ DNA.

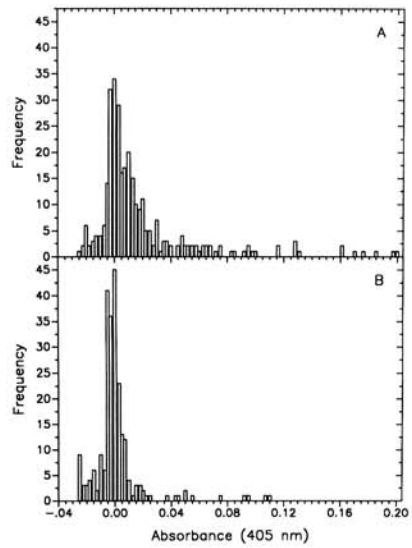

Fig. 3. Frequency distribution of samples tested by enzyme-linked immunosorbent assay (ELISA) for bacterial ring rot and giving absorbance readings <0.2. **(A)** Inoculated plants were given doses of 10^2 or 10^9 CFU of *Clavibacter michiganensis* subsp. *sepedonicus* prior to planting. **(B)** Control plants were mock inoculated with sterile phosphate buffer.

µl of PCR mix (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 200 mM dNTP, 1 mM primer CSRS-C, 20% deionized acetamide, 1.25 U AmpliTaq DNA polymerase [Perkin Elmer Cetus, Norwalk, CT] 3 mM $MgCl_2$, and 0.75% Tween 20) and overlaid with mineral oil. PCR reactions were cycled 36 times on a Perkin-Elmer DNA thermal cycler 480 under the following regime: cycle 1, 94°C/3 min, 50°C/ 60 s, 73°C/90 s; cycles 2 through 36, 94°C/60 s, 50°C/ 60 s, 73°C/90 s. PCR reaction products were then run on a 0.6% agarose gel, stained with ethidium bromide, and visualized with UV light.

Data analysis. Assay results were entered into the computer as binary response variables and compared using logical operators (e.g., and, if, or). Based on the recommendations of the manufacturer, an absorbance value of A_{405} = 0.2 was used as the threshold for ELISA samples to be scored as positive. PCR and DHA samples were scored as positive based on the presence of bands of appropriate size on agarose gels or distinct signals on autoradiograms, respectively. Overall performance of individual assays was compared using Cochran's test for related observations (4). The effect of inoculum dose, cultivar, location, and sampling date on individual assay performance was determined by analysis of covariance using PROC GLM in SAS (SAS Institute, Cary, NC). Unless otherwise noted, data presented herein represent final test results for all samples.

RESULTS AND DISCUSSION

Our PCR protocol used a 20-bp synthetic oligomer (CSRS-C) derived from the repeated sequence contained on a 1.078-kb *Sma*I fragment of the plasmid pCS1, which is contained in all strains of *C. michiganensis* subsp. *sepedonicus* tested except P40 (21,22). This repeated

Table 2. Comparison of enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), and DNA hybridization assay DHA) for the detection of bacterial ring rot of potato[a]

	Positive samples (%)											
	BelRus						Russet Burbank					
	North Dakota			New York			North Dakota			New York		
DAP/Assay	Buffer	10^2	10^9	Buffer	10^2	10^9	Buffer	10^2	10^9	Buffer	10^2	10^9
35 DAP												
ELISA	0.0	3.3	70.0	0.0	0.0	10.0	0.0	31.0[b]	83.3	0.0	6.7	23.3
PCR	0.0	3.3	70.0	0.0	0.0	6.7	0.0	27.6[b]	83.3	0.0	3.3	20.0
DHA	0.0	0.0	30.0	0.0	0.0	6.7	0.0	3.4[b]	40.0	0.0	3.3	16.7
DHA (frozen)	0.0	3.3	70.0	0.0	0.0	6.7	0.0[b]	20.7[b]	80.0	0.0	3.3	10.0
90 DAP												
ELISA	0.0	0.0[b]	90.0	0.0	0.0	60.0	0.0[b]	20.0	90.0	0.0[b]	16.7	66.7
PCR	0.0	0.0[b]	93.3	0.0	0.0	66.7	0.0[b]	20.0	93.3	0.0[b]	20.0	70.0
DHA	0.0	0.0[b]	93.3	0.0	0.0	73.3	0.0[b]	20.0	96.7	0.0[b]	16.7	63.3
DHA (frozen)	0.0	0.0[b]	86.7	0.0	0.0	56.7	0.0[b]	13.3	93.3	0.0[b]	3.3	53.3

[a] Percent of 30 samples (except those marked with footnote b). Plants were inoculated with buffer (control), 10^2, or 10^9 CFU *Clavibacter michiganensis* subsp. *sepedonicus* prior to planting and harvested at 35 or 90 days after planting (DAP). Overall differences were significant for each assay by analysis of covariance, P = 0.0001 for each assay. R^2 = 0.943, 0.948, 0.962, 0.955; MSE = 0.045, 0.044, 0.028, and 0.035 for ELISA, PCR, DHA, and DHA (frozen), respectively. Location, cultivar, sampling date, and inoculum dose main effects were significant for all assays, $P \leq 0.05$.

[b] Percent of 29 samples.

Table 3. Mean absorbance values for bacterial ring rot samples tested with enzyme-linked immunosorbent assay (ELISA)

				Samples with $A_{405} > 0.1$			Samples with $A_{405} > 0.2$		
Location	Cultivar	Dose[a]	Date[b]	Mean	Standard deviation	N[c]	Mean	Standard deviation	N[c]
North Dakota	BelRus	0	35	ND[d]	ND	ND	ND	ND	ND
North Dakota	BelRus	10^2	35	0.500	0.000	1	0.500	0.000	1
North Dakota	BelRus	10^9	35	0.900	0.318	21	0.900	0.318	21
North Dakota	BelRus	0	90	ND	ND	ND	ND	ND	ND
North Dakota	BelRus	10^2	90	ND	ND	ND	ND	ND	ND
North Dakota	BelRus	10^9	90	0.449	0.157	28	0.459	0.151	27
North Dakota	Russet Burbank	0	35	ND	ND	ND	ND	ND	ND
North Dakota	Russet Burbank	10^2	35	0.952	0.227	9	0.952	0.227	9
North Dakota	Russet Burbank	109	35	1.138	0.331	26	1.178	0.266	25
North Dakota	Russet Burbank	0	90	ND	ND	ND	ND	ND	ND
North Dakota	Russet Burbank	10^2	90	0.445	0.111	6	0.445	0.111	6
North Dakota	Russet Burbank	10^9	90	0.757	0.315	28	0.779	0.299	27
New York	BelRus	0	35	ND	ND	ND	ND	ND	ND
New York	BelRus	10^2	35	ND	ND	ND	ND	ND	ND
New York	BelRus	10^9	35	0.661	0.416	3	0.661	0.416	3
New York	BelRus	0	90	ND	ND	ND	ND	ND	ND
New York	BelRus	10^2	90	ND	ND	ND	ND	ND	ND
New York	BelRus	10^9	90	0.547	0.253	21	0.589	0.226	19
New York	Russet Burbank	0	35	ND	ND	ND	ND	ND	ND
New York	Russet Burbank	10^2	35	0.536	0.245	2	0.536	0.245	2
New York	Russet Burbank	10^9	35	0.469	0.256	10	0.604	0.163	7
New York	Russet Burbank	0	90	ND	ND	ND	ND	ND	ND
New York	Russet Burbank	10^2	90	0.491	0.323	6	0.565	0.298	5
New York	Russet Burbank	10^9	90	0.598	0.276	24	0.689	0.199	20

[a] In CFU.

[b] Sampling date (days after planting)

[c] Number of samples that make up the mean.

[d] None of the samples in this treatment exceeded the ELISA thresholds of A_{405} = 0.1 or 0.2.

sequence contains an inverted repeat and, therefore, requires only a single primer for amplification. The primer binds to nucleotides 20 to 39 of the *Sma*I fragment (and the homologous region of the complementary strand) and, when amplified, produces a 1.054-kb product that represents residues 20 to 1073 of the original *Sma*I fragment. This primer is derived from the same DNA sequence used in previous DHA studies (12,30).

The results obtained with tests of pure bacterial cultures are similar to those previously reported for DHA (12). Positive PCR results were obtained with all of the *C. michiganensis* subsp. *sepedonicus* strains tested (Table 1). With the exception of four strains of *C. michiganensis* subsp. *insidiosus*, none of the other coryneform bacteria gave results comparable to that expected for a positive PCR test for bacterial ring rot. PCR of some coryneform bacteria, as well as some of the other bacteria tested, resulted in nonspecific PCR products that were clearly distinguishable from that produced by *C. michiganensis* subsp. *sepedonicus* based on differences in size and/or the number of bands present on gels (Fig. 1). The production of nonspecific PCR products in tests of *Curtobacterium, Erwinia,* and *Pseudomonas* has previously been reported (23).

When dilution series of pure cultures of *C. michiganensis* subsp. *sepedonicus* were tested, we found that PCR was more sensitive than other assays that are commonly used for the diagnosis of bacterial ring rot (30). In these tests, PCR routinely detected fewer than 10 CFU per reaction (Fig. 2). At extremely high dilutions of pure cultures (i.e., <1 CFU per reaction), results were inconsistent, presumably due to the low probability of actually having target DNA present in the reaction mixture. PCR appears not to be affected by *C. michiganensis* subsp. *sepedonicus* strain as we were able to detect both fluidal and nonfluidal strains of this bacterium with equal sensitivity (Fig. 2). This is in contrast to reports that have shown that some serological detection methods are dependent upon the production of extracellular antigen by *C. michiganensis* subsp. *sepedonicus* (1,2).

Initial development of the protocol for testing crude plant materials revealed that the concentrations of $MgCl_2$ and SDS in the PCR reaction mix were critical to the success of this assay. It was also found that dilution of the crude sample was necessary to eliminate inhibition of the PCR reaction. Using a protocol optimized for testing crude plant materials, PCR proved to be a specific and sensitive assay for testing field samples. *C. michiganensis* subsp. *sepedonicus* was detected by all of the assay methods used in this study (Table 2), with a total of 24.2 % of the 716 samples tested giving positive PCR tests, versus 23.9 and 20.9% for ELISA and DHA, respectively (36.2, 35.8, and 29.1% of inoculated samples for PCR, ELISA, and DHA, respectively). Overall, the differences observed in assay performance were statistically significant (Cochran's test, $P < 0.0001$), with both PCR and ELISA having a significantly higher rate of detection than DHA (Cochran's test, $P \leq 0.001$). Overall, there was a slight, but insignifi-

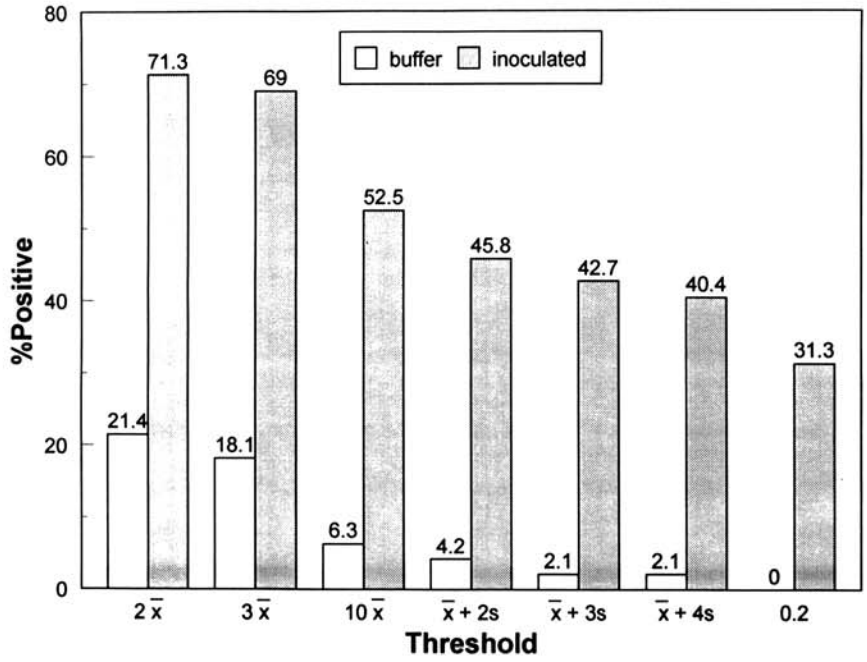

Fig. 4. Effect of positive-negative threshold on detection of *Clavibacter michiganensis* subsp. *sepedonicus* by enzyme-linked immunosorbent assay (ELISA). The mean ($\bar{x}$) and standard deviation (s) of absorbance values for buffer inoculated plants were 0.002 and 0.018, respectively.

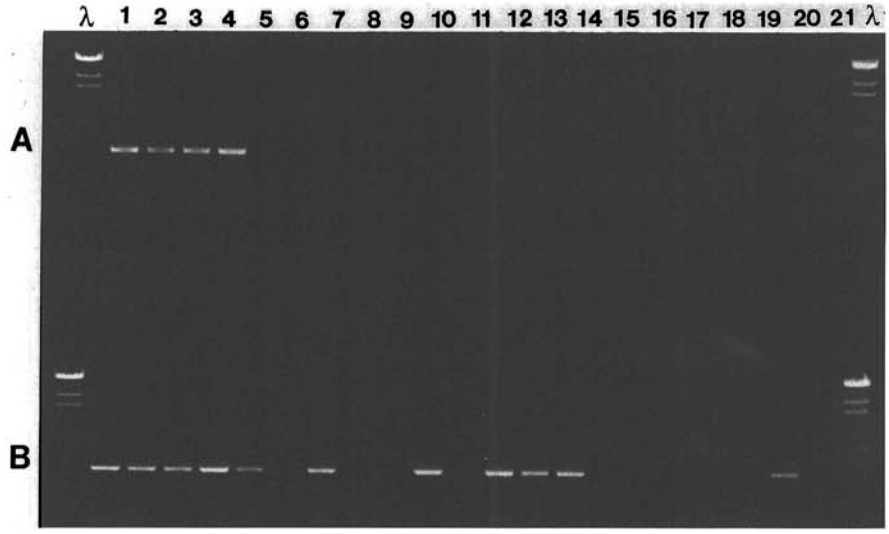

Fig. 5. Polymerase chain reaction (PCR) product resulting from tests of field-grown potato stems with primer CSRS-C. Seed of potato cvs. BelRus and Russet Burbank was inoculated with 0.05 M phosphate buffer pH 7.2 (control) or 10^2 or 10^9 CFU of *Clavibacter michiganensis* subsp. *sepedonicus* strain SS43. All samples were grown near Fargo, ND, and were harvested at 35 days after planting. PCR product was run on 0.6% agarose and stained with ethidium bromide. Row A: lanes 1 to 5, BelRus, 10^9 CFU; lanes 6 to 10, BelRus, 10^2 CFU; lanes 11 to 15, Russet Burbank control; lanes 16 to 20, BelRus control. Row B: lanes 1 to 5, Russet Burbank, 10^9 CFU; lanes 6 to 9, Russet Burbank, 10^2 CFU; lanes 10 to 14 BelRus, 10^9 CFU; lanes 15 to 19 Russet Burbank control; lane 20, *C. m.* subsp. *sepedonicus* strain SS43 (positive control); lane 21, Tris-EDTA (TE) buffer (negative control). Marker (λ) is *Bam*HI-digested λ DNA.

Table 4. Percent agreement among enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) test, and DNA hybridization assay (DHA) results for bacterial ring rot samples[a]

	PCR	DHA (fresh)	DHA (frozen)
ELISA	96.9	90.8	96.5
PCR	–	89.9	96.2
DHA	–	–	90.9

[a] Total of 716 samples per assay.

cant (Cochran's test, $P = 0.1859$) increase in the number of positives (31.4 versus 29.1% of inoculated samples) obtained with DHA when the samples were frozen prior to blotting on nylon membranes, presumably due to an enhanced release of bacteria from plant tissues.

The experimental design of the field plot was such that it allowed evaluation of the assays over a wide range of conditions that would be confronted by a seed inspector and/or diagnostician and included a "best case, worst case" scenario, i.e., the combination of a high inoculum dose, susceptible cultivar (Russet Burbank), and late sampling date versus the combination of a low inoculum dose, tolerant cultivar (BelRus), and early sampling date. Each of the assays used in this study was significantly affected by inoculum dose, cultivar, and sampling date (by analysis of covariance, $P = 0.0001$ for each assay) (Table 2), which confirms the results of a previous study (12). When these factors were taken into account, the general trend for greater frequency of detection with PCR than with the other assays remained. In the "best case" scenario, *C. michiganensis* subsp. *sepedonicus* was detected in 70 and 93.3% of samples from New York and North Dakota, respectively, while in the "worst case" scenario, detection was low and, in many cases, the bacterium was not detected at all. The low rates of detection observed for some treatment combinations are of concern. However, it is unclear whether the negative test results represented an inability to detect low bacterial populations in plants or whether they resulted from a lack of infection following inoculation. The data suggest that the latter explanation may account for many of these negative tests. The majority of the control plants (96%) gave ELISA values in the range $-0.025 \leq A_{405} \leq 0.038$ (Fig. 3). Eighty-four percent of the inoculated plants that were negative by ELISA had absorbance values in that range and, therefore, are likely to be true negatives. Previous studies (7,15) have shown that bacterial populations in infected plants reach detectable levels early in the growing season, an observation confirmed by our ability to detect the bacterium at 35 DAP. For many of the treatment combinations included in this study (e.g., North Dakota samples and BelRus plants given low inoculum doses), however, the number of positives did not increase substantially as the growing season progressed, suggesting that infected plants tended to develop detectable populations. Moreover, there was no apparent relationship between inoculum dose, location, cultivar, or sampling date and bacterial populations in plants that were infected (Table 3). This is in contrast to previously published studies reporting that tolerant cultivars support lower populations of *C. michiganensis* subsp. *sepedonicus* (7,8,15) and may indicate some resistance to infection in these cultivars. Differences in our ability to detect *C. michiganensis* subsp. *sepedonicus* between locations may indicate that resistance to infection can be modulated by environmental conditions and may help explain environmental effects upon symptom expression (29).

Although PCR tended to have higher rates of detection than ELISA, this difference was not statistically significant (Cochran's test, $P = 0.5639$). However, the performance of ELISA was found to be highly dependent upon the threshold that was used for the characterization of samples as positive or negative (Fig. 4). As De Boer (6) has noted, samples tested for bacterial ring rot with ELISA tend to form a continuum between low and high absorbance readings and do not display the bimodal distribution that is considered to be optimum for determining a threshold value. In this study, considerable overlap between the absorbance values obtained for control and inoculated plants was observed (Fig. 3). If the threshold value selected was too low, several control plants would be scored as positive (Fig. 4). The use of a higher threshold, (i.e., $A_{405} = 0.2$) completely eliminated the problem of scoring control plants as positive but increased the number of potential false

Table 5. Results of retesting of bacterial ring rot samples giving equivocal results with polymerase chain reaction (PCR)[a]

Initial assay results							
ELISA[b]	PCR	PCR retest	Sample No.	Location	Cultivar	Dose[c]	Date[d]
1.142 (+)	–	+	3	North Dakota	Russet Burbank	10^2	35
0.997 (+)	–	+	57	North Dakota	BelRus	10^9	35
0.962 (+)	–	+	2	North Dakota	Russet Burbank	10^2	35
0.802 (+)	–	+	304	North Dakota	Russet Burbank	10^9	90
0.793 (+)	–	+	301	North Dakota	Russet Burbank	10^9	90
0.757 (+)	–	+	108	North Dakota	Russet Burbank	10^9	35
0.659 (+)	–	+	572	New York	Russet Burbank	10^2	90
0.561 (+)	–	+	196	North Dakota	BelRus	10^9	90
0.506 (+)	–	+	107	North Dakota	Russet Burbank	10^9	35
0.493 (+)	–	+	625	New York	Russet Burbank	10^9	90
0.457 (+)	–	+	268	North Dakota	Russet Burbank	10^2	90
0.428 (+)	–	+	683	New York	BelRus	10^9	90
0.417 (+)	–	+	263	North Dakota	Russet Burbank	10^9	90
0.305 (+)	–	+	273	North Dakota	BelRus	10^9	90
0.263 (+)	–	+	291	North Dakota	Russet Burbank	10^2	90
0.232 (+)	–	+	649	New York	Russet Burbank	10^9	90
0.601 (+)	–	–	385	New York	Russet Burbank	10^9	35
0.548 (+)	–	–	172	North Dakota	Russet Burbank	10^2	35
0.363 (+)	–	–	449	New York	Russet Burbank	10^2	35
0.259 (+)	–	–	544	New York	Russet Burbank	10^2	90
0.205 (+)	–	–	417	New York	BelRus	10^9	35
0.178 (–)	+	+	647	New York	Russet Burbank	10^9	90
0.161 (–)	+	+	232	North Dakota	Russet Burbank	10^9	90
0.117 (–)	+	+	652	New York	Russet Burbank	10^2	90
0.031 (–)	+	+	651	New York	Russet Burbank	10^2	90
0.004 (–)	+	–	720	New York	BelRus	10^9	90
0.187 (–)	–	+	272	North Dakota	BelRus	10^9	90
0.200 (–)	–	+	603	New York	BelRus	10^9	90

[a] Total samples tested = 716.
[b] A_{405} scores for samples tested by enzyme-linked immunosorbent assay (ELISA). Sign in parentheses indicates whether sample was scored as positive (+) or negative (–) based on a threshold value of $A_{405} = 0.2$.
[c] In CFU.
[d] Sampling date (days after planting).

negative tests. In contrast, both PCR and DHA results tended to be clear cut, with either a strong or no signal observed in agarose gels or autoradiograms, respectively. Only the 1.054-kb PCR product expected for *C. michiganensis* subsp. *sepedonicus* was observed in tests of plant samples (Fig. 5) and none of the buffer-inoculated plants tested positive with either PCR or DHA.

There was general agreement among the assays used in this study (Table 4), indicating that unequivocal test results could be obtained for the majority of the samples tested. These results were, in general, confirmed in cases in which samples were retested. However, PCR results for 28 samples (3.6% of total) did not agree with those obtained using ELISA (Table 5). The majority of these samples (21/28) initially were positive by ELISA and negative by PCR. Upon retesting aliquots of the original sample extracts, 16 of these samples were positive by PCR. Of five samples that tested negative by ELISA and positive by PCR, four were PCR-positive when retested. These equivocal test results might be explained by the low probability of including target DNA in the PCR reaction mix due to low bacterial populations or difficulty in extracting DNA from the original sample. The last three samples in Table 5, however, are of concern as they may represent false positive tests. These observations underscore the importance of multiple testing of individual samples and the use of independent confirmatory assays for the diagnosis of bacterial ring rot (18,30). They also indicate that, as assay sensitivity ceases to be a limiting factor, the accuracy and reproducibility of test results may become more dependent upon appropriate sample selection and processing than upon any inherent limitation(s) of the assay itself. Ironically, since the sensitivity of PCR exceeds that of currently available assays, independent confirmation of equivocal PCR test results may be difficult and may prove to be the major challenge associated with this assay. Utilization of multiple primer sets for PCR assays may be an approach to resolve this potential problem.

In summary, PCR appears to have potential as a sensitive and specific assay for bacterial ring rot. Our results show that, in terms of frequency, detection of *C. michiganensis* subsp. *sepedonicus* in crude plant extracts by PCR is at least equal to ELISA and superior to DHA. The two DNA-based assays have the additional advantage of not relying on an arbitrary positive threshold, and apparently have greater specificity since none of the control plants gave positive tests with either PCR or DHA.

ACKNOWLEDGMENTS

Research supported in part by Cornell Hatch project NYC-153406 and by USDA-ARS Potato Grant 58-1275-1-142.

LITERATURE CITED

1. Baer, D., and Gudmestad, N. C. 1993. Serological detection of nonmucoid strains of *Clavibacter michiganensis* subsp. *sepedonicus* in potato. Phytopathology 83:157-163.
2. Bishop, A. L., Clarke, R. G., and Slack, S. A. 1988. Antigenic anomaly in a naturally occurring nonfluidal strain of *Corynebacterium sepedonicum*. Am. Pot. J. 65:237-246.
3. Bishop, A. L., and Slack, S. A. 1987. Effect of inoculum dose and preparation, strain variation, and plant growth conditions on the eggplant bioassay for bacterial ring rot. Am. Pot. J. 64:227-234.
4. Conover, W. J. 1980. Practical Nonparametric Statistics. John Wiley and Sons, New York.
5. Crowley, C. F., and De Boer, S. H. 1982. Nonpathogenic bacteria associated with potato stems cross-react with *Corynebacterium sepedonicum* antisera in immunofluorescence. Am. Pot. J. 59:1-8.
6. De Boer, S. H. 1991. Current status and future prospects of bacterial ring rot testing. Am. Potato J. 68:107-113.
7. De Boer, S. H., and McCann, M. 1989. Determination of population densities of *Corynebacterium sepedonicum* in potato stems during the growing season. Phytopathology 79:946-951.
8. De Boer, S. H., and McCann, M. 1990. Detection of *Corynebacterium sepedonicum* in potato cultivars with different propensities to express ring rot symptoms. Am. Potato J. 67:685-694.
9. De Boer, S. H., and Slack, S. A. 1984. Current status and prospects for detecting and controlling bacterial ring rot of potatoes in North America. Plant Dis. 68:841-844.
10. De Boer, S. H., Wieczorek, A., and Kummer, A. 1988. An ELISA test for bacterial ring rot of potato with a new monoclonal antibody. Plant Dis. 72:874-878.
11. Drennan, J. L., Slack, S. A., Westra, A. A. G., Collmer, A., Gudmestad, N. C., and Oleson, A. E. 1994. Specific detection of *Clavibacter michiganensis* subsp. *sepedonicus* with the polymerase chain reaction. (Abstr.) Phytopathology 84:1087.
12. Drennan, J. L., Westra, A. A. G., Slack, S. A., Delserone, L. M., Collmer, A., Gudmestad, N. C., and Oleson, A. E. 1993. Comparison of a DNA hybridization probe and ELISA for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* in field-grown potatoes. Plant Dis. 77:1243-1247.
13. Firrao, G. 1990. Cloned diagnostic probe for *Clavibacter michiganensis* ssp. *sepedonicus*. EPPO Bull. 20:207-213.
14. Glick, D. P., Ark, P. A., and Racicot, H. N. 1944. Outline of the procedure for the diagnosis of bacterial ring rot of potatoes - A report to the Certification Committee of the Potato Association of America. Am. Potato J. 21:311-314.
15. Gudmestad, N. C., Baer, D., and Kurowski, C. J. 1991. Validating immunoassay test performance in the detection of *Corynebacterium sepedonicum* during the growing season. Phytopathology 81:475-480.
16. Johansen, I. E., Rasmussen, O. F., and Heide, M. 1989. Specific identification of *Clavibacter michiganense* subsp. *sepedonicum* by DNA-hybridization probes. Phytopathology 79:1019- 1023.
17. Li, X., and De Boer, S. H. 1995. Selection of polymerase chain reaction primers from an RNA intergenic spacer region for specific detection of *Clavibacter michiganensis* subsp. *sepedonicus*. Phytopathology 85:837-842.
18. Manzer, F. E., and Slack, S. A. 1979. Report of the Pathology Section committee on bacterial ring rot diagnosis. Am. Potato J. 56:551-555.
19. Miller, H. J. 1984. Cross-reactions of *Corynebacterium sepedonicum* antisera with soil bacteria associated with potato tubers. Neth. J. Plant Pathol. 90:23-28.
20. Mirza, M. S., Rademaker, J. L. W., Janse, J. D., and Akkermans, A. D. L. 1993. Specific 16S ribosomal RNA targeted oligonucleotide probe against *Clavibacter michiganensis* subsp. *sepedonicus*. Can. J. Microbiol. 39: 1029-1034.
21. Mogen, B. D., and Oleson, A. E. 1987. Homology of pCS1 plasmid sequences with chromosomal DNA in *Clavibacter michiganense* subsp. *sepedonicum*: Evidence for the presence of a repeated sequence and plasmid integration. Appl. Environ. Microbiol. 53: 2476-2481.
22. Mogen, B. D., Oleson, A. E., Sparks, R. B., Gudmestad, N. C., and Secor, G. A. 1988. Distribution and partial characterization of pCS1, a highly conserved plasmid present in *Clavibacter michiganense* subsp. *sepedonicum*. Phytopathology 78:1381-1386.
23. Rademaker, J. L. W., and Janse, J. D. 1994. Detection and identification of *Clavibacter michiganensis* subsp. *sepedonicus* and *Clavibacter michiganensis* subsp. *michiganensis* by nonradioactive hybridization, polymerase chain reaction, and restriction enzyme analysis. Can. J. Microbiol. 40:1007-1018.
24. Rademaker, J. L. W., Thalen, M., and Janse, J. D. 1992. Experiences with DNA-Hybridization using a biotinylated probe against *Clavibacter michiganensis* subsp. *sepedonicus*. Meded. Fac. Landbouwwet. Rijksuniv. Gent. 57:263-268.
25. Schnieder, B. J., Zhao, J., and Orser, C. S. 1993. Detection of *Clavibacter michiganensis* subsp. *sepedonicus* by DNA amplification. FEMS Microbiol. Lett. 109:207-212.
26. Slack, S. A., Kelman, A., and Perry, J. B. 1978. Comparison of three seriodiagnostic assays for the detection of *Corynebacterium sepedonicum*. Phytopathology 69:186-189.
27. Slack, S. A., Sanford, H. A., and Manzer, F. E. 1979. The latex agglutination test as a rapid serological assay for *Corynebacterium sepedonicum*.. Am. Potato J. 56:441-446.
28. Vidaver, A. 1967. Synthetic and complex media for the rapid detection of fluorescence of phytopathogenic pseudomonads: Effect of the carbon source. Appl. Microbiol. 15:1523-1524.
29. Westra, A. A. G., and Slack, S. A. 1994. Effect of interaction of inoculum dose, cultivar, and geographic location on the magnitude of bacterial ring rot symptom expression in potato. Phytopathology 84: 228-235.
30. Westra, A. A. G., Slack, S. A., and Drennan, J. L. 1994. Comparison of some diagnostic assays for bacterial ring rot of potato: a case study. Am. Potato J. 71:557-565.