PCR Detection of *Clavibacter michiganensis* Subsp. *sepedonicus*-infected Tuber Samples in a Plate Capture Assay

Debra Baer[1], Emily Mitzel[2], Julie Pasche, and Neil C. Gudmestad*

Department of Plant Pathology, North Dakota State University, Fargo, North Dakota 58105
[1]Present address of first author: North Dakota State Seed Department, P.O. Box 5257, Fargo, ND 58105.
[2]Present address of second author: Cargill, Inc., Central Research, 2301 Crosby Road, Wayzata, MN 55391
*Corresponding author: Tel: (701) 231-7547; Fax: (701) 231-7851; Email: Neil_Gudmestad@ndsu.nodak.edu.

ABSTRACT

The speed and sensitivity of PCR-based assays allow shorter turnaround times for the detection of pathogens for which culture and serological methods are difficult or unavailable. PCR was performed with primer sets Cms50 and Cms72, designed previously by Mills *et al.* (1997) through subtractive hybridization to detect *Clavibacter michiganensis* subspecies *sepedonicus* (*Cms*). In bacterial suspensions, fewer than three cells/10 ul reaction were detected after PCR amplicons were hybridized with specific DIG-labeled DNA probes in an enzyme-linked oligonucleosorbent assay (ELOSA). In naturally infected tuber samples representing three cultivars of potato, the diagnostic sensitivity of PCR/ ELOSA was 96%, while the specificity exceeded 99%. PCR/ELOSA detected *Cms* in infected tuber samples with equal sensitivity regardless of colony morphology, potato cultivar, or primer sets.

INTRODUCTION

Current advances in the application of DNA hybridization and polymerase chain reaction (PCR) for detection of pathogenic microorganisms has led to the development of several *Cms* DNA detection methods (Drennan *et al.* 1993; Li and DeBoer 1995; Rademaker and Janse 1994; Schneider *et al.* 1993). The DNA hybridization method, utilizing the repeated DNA sequence (RS) probe, provided a detection limit of 10^5 to 10^6 *Cms* bacteria, and no hybridizations occurred with healthy potato stems, tubers, or any cross-reacting bacteria (Drennan *et al.* 1993). The major problem with the RS probe is that it is not useful against all *Cms* strains because the plasmid pCS1 is absent in one strain, CsP45 (Mogen *et al.*1988). Also, the RS probe was found to have homology with eight of 11 strains of an alfalfa pathogen, *C. m.* subsp. *insidious* (Drennan *et al.*1993). PCR probes made from other *Cms* DNA sequences lacked specificity or failed to detect all isolates (Rademaker and Janse 1994). Li and De Boer (1995) synthesized PCR primers that anneal to the variable region within the intergenic spacer (ITS) region of the rDNA repeat of *Cms*. This primer set was reported to have 95% homology with the ITS of other subspecies. Although the ITS was amplified only from strains of *Cms*, it differed at only two or three bases with respect to analogous regions in four closely related subspecies; requiring more rigid control of the annealing temperature to maintain primer specificity.

Three primer sets, designated Cms50, Cms72, and Cms85, were developed for the detection of *Cms* (Mills *et al.*1997). The three single-copy, unique DNA fragments selected by subtraction hybridization from *Cms* strain CS3, were previously found to have absolute specificity for 29 mucoid, intermediate, and nonmucoid strains of *Cms* bacteria *in vitro* when used in polymerase chain reaction (PCR) amplification, and did not hybridize with non-*Cms* strains (Mills *et al.* 1997). The detection level was approximately 100 colony-forming units (cfu)/ml (Mills *et al.* 1997). Complementary biotinylated capture probes, Cp50,

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Cp72, and Cp85, were generated for enzyme-linked oligonucleosorbent assay (ELOSA) evaluation of amplified PCR products.

The success of PCR amplification is usually monitored by agarose gel electrophoresis of ethidium bromide-stained amplified DNA fragments. As an alternative to this step, a colorimetric method for the detection of PCR products was used. This approach to visualize the DNA products is based on initial amplification of target DNA followed by ELOSA. ELOSA is similar to ELISA, but rather than using antigens and antibodies, the assay uses DNA-DNA hybridization. In ELOSA, the PCR product is labeled with a digoxigenin (DIG) reporter group by incorporating DIG-labeled dUTP's in the PCR reaction. ELOSA relies on annealing of separate biotinylated and digoxigenated probes to the amplified BRR nucleic acid; these complexes are captured on streptavidin-coated micro titer plates and detected using an anti-digoxigen antibody conjugate. When the substrate for the enzyme attached to the anti-DIG antibody is added a color change occurs, which then can be measured and quantified by standard spectrophotometry. ELOSA is more expensive than gel electrophoresis, but it is faster and easier to quantitate many samples using a microplate reader. ELOSA is also a safer procedure because it does not utilize ethidium bromide.

The objective of this study was to determine the feasibility of PCR in post-harvest screening of certified seed potatoes for *Cms* using two primers isolated from *Cms* strain CS3 by Mills *et al.* (1997). In this study we evaluated five additional *in vitro Cms* strains and five *Clavibacter* species not previously reported for these primer sets and hybridization probes. We then evaluated the same *Cms* strains in naturally infected tuber tissue and compared the standard PCR methods using gel electrophoresis to PCR products evaluated with ELOSA.

TABLE 1—*Plant pathogenic* Clavibacter *and* Erwinia *species used to test for specificity.*

Organism	Source
Clavibacter toxicosa	
NZ 49908	1- USA
Rathayibacter rathayi	
ATCC 13659	2
Clavibacter xyli	
TB 1A M.	J. Davis – China
Clavibacter tritici	
NZ 2623	1 – India
Clavibacter iranicus	
NZ 3496	1
C. michiganensis subsp. *tessellarius*	
NZ 7219	1 – USA
Erwinia carotovora subsp. *carotovora* #6	N. C. Gudmestad- USA
C. michiganensis subsp. *michiganensis*	
NZ 2539	1
NZ 2541	1 – U. K.
NZ 2545	1 – Sicily
NZ 2550	1– Hungary
C. michiganensis subsp. *insidiosus*	
NZ 3567	1 – Australia
NZ 3619	1
NZ 4543	1
NZ 4191	1
C. michiganensis subsp. *nebraskensis*	NZ 5366 1 – USA
NZ 5367	1 – USA
NZ 5368	1 – USA
NZ 5369	1 – USA

[1] New Zealand Plant Disease Division Culture Collection, Auckland, New Zealand.
[2] American Type Culture Collection, Rockville, MD.

MATERIALS AND METHODS

In Vitro *Bacterial Studies*

Cms isolates representing mucoid (OFF, As-1), intermediate (Wi-2), and nonmucoid colony morphologies (SD-1, INM-1) were grown on NBY agar plates (Schaad 1988). A list of *Clavibacter* sp. and *Erwinia carotovora* strains used for specificity studies is provided in Table 1. Bacteria were cultured and plate counts were performed in duplicate as previously described (Bertoni and Mills 1987). Suspensions were adjusted to 10^8 cfu/ml and diluted 10-fold serially to 10^0. The total number of CFU in each dilution was recorded. Aliquots of dilutions containing 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 cfu/ml of each bacterial strain were tested with PCR to determine the dilution endpoint, specificity of primers to be used in the field study, and sensitivity to the strains of *Cms* used to inoculate seed tubers.

Inoculation of Field Samples

The production of field grown *Cms*-infected plant material for use in the laboratory was performed for two consecutive growing seasons. *Cms* strains OFF (mucoid), As-1 (mucoid), INM-1 (nonmucoid), Wi-2 (intermediate), and SD-1 (nonmucoid), were grown on NBY agar. The potato cultivars Russet Burbank, Norchip, and Red Norland were used to represent late, middle, and early maturing cultivars, respectively. Seed pieces were vacuum infiltrated as previously described (De Boer and McCann 1989). Each *Cms* strain was inoculated into potato seed pieces at 10^4 and 10^8 cfu/ml. Non-infected controls of each potato cultivar were vacuum infiltrated with ? strength nutrient broth. Seed pieces were placed in paper sacks with 25 g of fungicide

potato seed treatment (8% mancozeb/fir bark) and 250 g of vermiculite™ (American Vermiculite Corp., Kennesaw, GA) and planted 2 wk after vacuum infiltration. A total of 33 treatments containing 25 seed pieces per treatment were planted.

Immunofluorescent Assay

Indirect fluorescent assay staining (IFAS) was performed on macerated, soak fluid, and blender filtrate samples to determine *Cms* populations as immunofluorescent units per gram tuber tissue (IFU/g) as previously described (Baer and Gudmestad 1993) following standard procedures (DeBoer and Wieczorek 1984).

Post-harvest Sample Preparation

Tubers were harvested approximately 133 days after planting and stored at 11 C until processed. One random sample was selected from each treatment (inoculation level X *Cms* strain X potato cultivar). A tuber core sample (0.5 ± 0.1 g) previously determined to be *Cms*-infected using IFAS (Baer and Gudmestad 1993; DeBoer and Wieczorek 1984) was placed in a flask containing 99 cores from presumptively uninfected tubers and processed using the soak and blender methods, respectively, as previously described (Dinesen and DeBoer 1995). Results from this group of samples were termed "strain x cultivar" subset.

Three tuber-processing methods, macerated, soak fluid, and blender fluid, were evaluated in PCR testing. Results from this group of samples were termed "sample preparation" subset. Tubers from three different cultivars of potatoes infected with *Cms* strains OFF (mucoid) and INM-1 (nonmucoid) were chosen according to bacterial ring rot (BRR) symptoms. Tubers displaying severe, moderate, light, very light, and no symptoms were chosen for IFAS testing. One sample of each set of strain and cultivar, asymptomatic yet positive by IFAS, was chosen according to the IFU/g value. Individual samples (~ 1 g) were macerated 1:1 in sterile PBS and centrifuged (190 x *g* for 10 min). The soak and blender methods of preparation were also performed on the asymptomatic infected tuber cores at a ratio of 1:100 and 1:200 with healthy cores (Dinesen and De Boer 1995).

DNA Extraction and Amplification

Bacterial suspensions, soak fluid, blender filtrate, and macerated single tuber samples (300 ul), were prepared according to manufacturer's directions with InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA). The product was used directly for PCR or stored at -20 C. Before each use, the mixture was centrifuged at 12,000-17,000 x *g* for 2-3 min.

In the strain x cultivar study, IFAS was performed on the original tuber to verify the sample was a true positive. The uninfected tubers were treated in the same manner to assure a true negative sample. IFAS was also done on each blender and soak subsample (33 composite samples). In the sample preparation study, IFAS was also performed on the original macerated sample and the blender and soak subsamples. Preliminary PCR/gel electrophoresis studies revealed that up to half of the undiluted tissue samples, whether macerated, blender, or soak samples, usually were not amplified while the next two 10-fold dilutions of the crude sample would be amplified. This indicated that at least two 10-fold dilutions of tissue sample were necessary to eliminate inhibition of the PCR reaction, despite the use of InstaGene Matrix, and this finding has been confirmed elsewhere (Mills *et al.* 1997; Schaad *et al.* 1999; Slack *et al.*1996). For each InstaGene Matrix preparation, 10 ul of template was amplified using 0.5 U AmpliTaq Gold DNA Polymerase (Perkin Elmer, Branchburg, NJ) in the presence of 1 uM of primer set Cms50 (forward and reverse) or 0.5 uM Cms72 (forward and reverse), 0.2 mM dATP, dCTP, dGTP, dTTP, 0.01 mM DIG-11dUTP (Roche, Indianapolis, IN), Gene Amp PCR buffer II (Perkin Elmer) (final concentration: 10 mM Tris-HCl, pH 8.3, 50 mM KCl). $MgCl_2$ was 2.5 mM in the final concentration. The total reaction volume was 25 ul.

Amplifications were carried out in a Peltier Thermal Cycler model PTC-100™ (M J Research, Watertown, MA) using PCR tubes specific for that cycler (M J Research). Target DNA was amplified for 10 cycles of 60 s at 94 C, 60 s at 63 C, and 10 s at 72 C, followed by 40 cycles of 35 s at 94 C, 35 s at 63 C, and 15 s at 72 C, then 1 cycle of 60 s at 72 C. Samples were held at 4 C until removed from the thermal cycler. PCR products were evaluated by gel electrophoresis or ELOSA.

PCR Product Evaluation with Gel Electrophoresis

Ten microliters of each PCR product were electrophoresed through a 2% agarose (low EEO, Sigma) (80 min, 120V) in 1x Tris base, Boric acid, and EDTA (TBE) buffer and stained with ethidium bromide (EthBr). MspI-digested pBR322 (New England Biolabs, Beverly, MA) was used as a size marker. Gels were photographed on a UV transilluminator Model TM-40 (UVP, Inc., Upland, CA) with a Polaroid MP-4 land camera using Polaroid professional #667 black and white instant pack film, ISO 3000 (Polaroid Corp., Ventura, CA).

PCR Product Evaluation with ELOSA

PCR product (25 ul) was diluted in hybridization solution (Na_2HPO_4, 0.25 M; NaCl, 0.25 M; 0.5 M EDTA, 1 mM) to 50 µl. The capture probe, Cp50 or Cp72, corresponding to the primer set used, was added at 25 ng. DNA was denatured for 10 min at 95 C and primers were annealed for 1 hr at 61 C. An additional 150 µl of hybridization solution was added to each tube; 100 µl of this solution was placed in duplicate wells of a streptavidin-coated plate (Roche). The plates were incubated for 1 hr at 37 C, then washed five times with PBST (PBS + 0.1% Tween 20, pre-heated to 37 C). Anti-digoxigenin-peroxidase, Fab fragments (Anti-dig POD)(Roche), (0.2 U/ml in PBST, 100 µl) were added to each well. The plates were incubated (1 hr, 37 C) and washed five times (PBST, 37 C). The substrate, 2,2'-azino-bis (3-ethylbenzthiazolline-6-sulfonic acid) (ABTS)(Roche), was added (100 µl) to each well of the micro titer plate. Absorbance (A_{405}) was read on an automated microplate reader (EL311, Bio-Tek Instruments, Inc.). The decision level for positive ELOSA results was two times the standard deviation above the mean absorbance (A_{405}) of the uninfected controls.

Data Analysis

Data analysis was performed by analysis of variance (ANOVA) using the Statistical Analysis System (SAS Institute, Inc, Cary, NC). A main effects model that analyzes a single variable at a time was used. Mean separation was performed by least significant differences (LSD). Data were entered into the ANOVA program in true positive numbers without transformation to evaluate the percentages of sensitivity. Data with a Pr>F value of 0.001 were considered statistically significant. Sensitivity, specificity, and efficiency were calculated as previously described (Baer and Gudmestad 1993).

Variables in subset strain x cultivar included five *Cms* strains (OFF, As-1, Wi-2, INM-1, and SD-1), three potato cultivars (Norchip, Red Norland, and Russet Burbank), two dilutions (10^{-1}, 10^{-2}), two primers (Cms50 and Cms72), two processing methods (soak fluid and blender filtrate), and two testing procedures (PCR evaluated with gel electrophoresis and PCR evaluated with ELOSA). Variables used in statistical analysis of sample preparation subset included two *Cms* bacterial strains (OFF and INM-1), three potato cultivars (Norchip, Red Norland, and Russet Burbank), two dilutions ($10^{-1}$and 10^{-2}), two primers (Cms50 and Cms72), five processing methods (macerated, soak fluid 1:100, blender filtrate 1:100, soak fluid 1:200, and blender filtrate 1:200), and two testing procedures (PCR evaluated with gel electrophoresis or ELOSA).

RESULTS

In Vitro *Bacterial Studies*

PCR Detection of Cms DNA in Bacterial Suspensions—Initial experiments were performed with the Cms50, Cms72, and Cms85 primers. In bacterial dilutions, only the Cms50 and Cms72 amplified *Cms* DNA consistently. We decided therefore to continue the investigations of the samples with the Cms50 and Cms72 primer sets only. To evaluate the sensitivity of our PCR amplification, 10-fold serial dilutions of each *Cms* strain were analyzed with the two primer sets. PCR amplification of all five *in vitro Cms* strains produced the expected DNA products of 195 base pairs (bp) for primer Cms50 and 164 bp for primer Cms72 after gel electrophoresis (Mills *et al.* 1997) (Table 2). *Cms* DNA in all five *Cms* strains could be detected after evaluating by gel electrophoresis (two replicates, three trials in three separate days). For all strains, the ELOSA assay appeared to be equal or greater in sensitivity when compared to gel electrophoresis for evaluating PCR products using either primer (Figure 1, Table 2). Equal sensitivities were achieved with the Cms50 and Cms72 primers, each detecting ≤3 colonies/reaction (cfu/rxn) according to the colony counts in each dilution. The primers Cms50 and Cms72 did not hybridize with any other bacterial pathogens of potato (*Erwinia*) or related *Clavibacter* species when PCR was performed in the same manner (Table 1). These *in vitro* studies prove that PCR/gel electrophoresis and PCR/ELOSA could be used to detect DNA from nonmucoid strains INM-1 and SD1 that are undetected by commercial ELISA kits.

Detection of Cms DNA in field infected tuber samples. All post-harvest samples of individual tubers collected from ring-rot-infected plants, (in both sample sets) in the form of undiluted soak fluid or blender filtrate, were at the lowest detection limit of IFAS. Population values in data set strain x cultivar ranged from 1.3×10^5 to 1.2×10^6 IFU/g in the blender fluid and 1.0×10^5 to 3.3×10^6 IFU/g in the soak fluid based on IFAS. These values corresponded to 0.6 to 4.7 IFU/high power field (hpf) and 0.4 to 1.3 IFU/hpf for blender and soak preparations, respectively. PCR amplification produced the expected DNA products of 195 bp and 164 bp for Cms50 and Cms72, respectively, from both the soak and blender methods of preparation.

Detection sensitivities in the strain x cultivar data subset were significantly different (Pr>F = 0.05) for *Cms* strain (Pr>F = 0.0001) and PCR product evaluation (Pr>F = 0.0001) (Table 3). The level of detection among *Cms* strains was significantly different; however, the detection level between the mucoid and

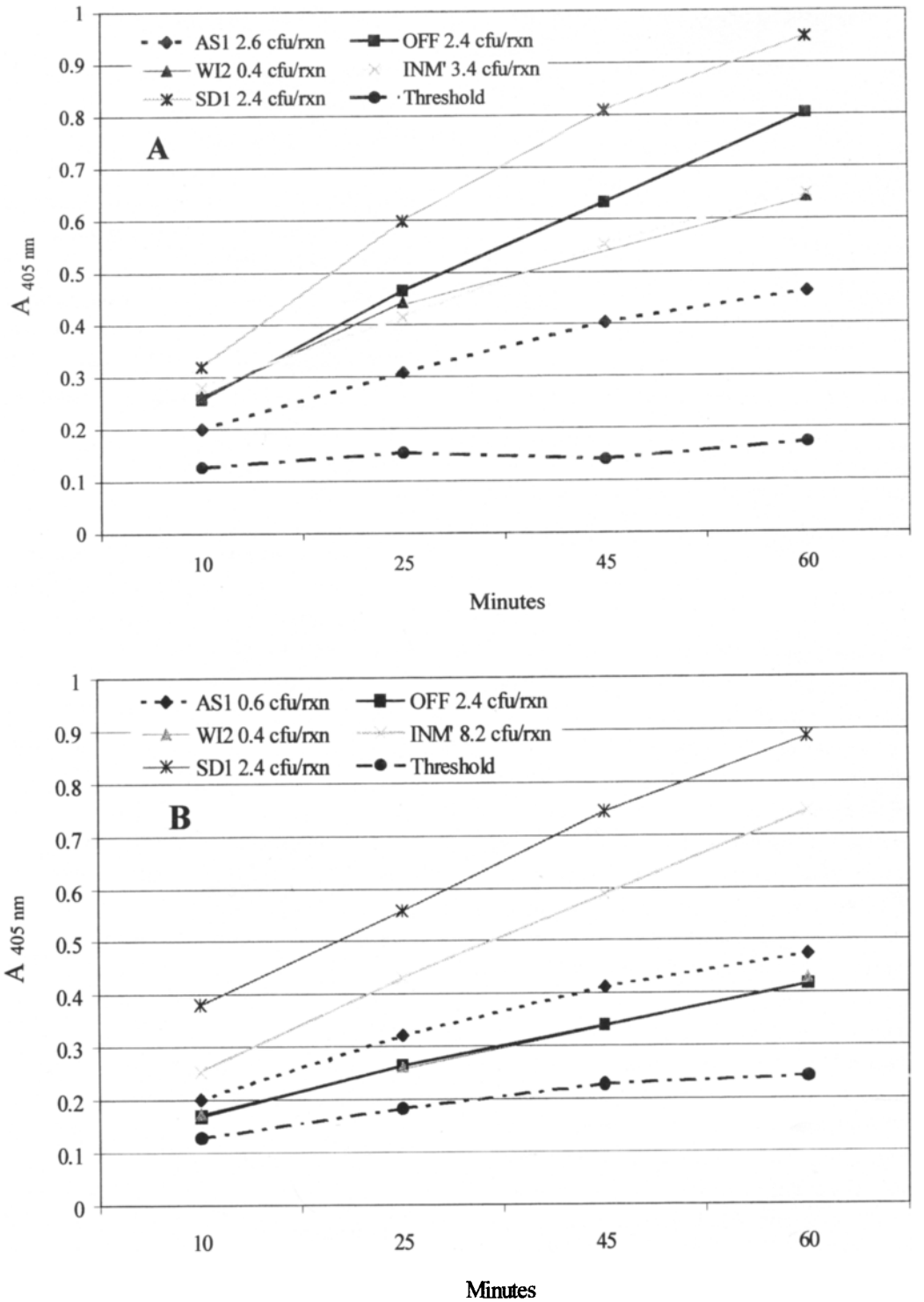

FIGURE 1.
Amplification of DNA of five known strains of *Clavibacter michiganensis* subsp. *sepedonicus* by polymerase chain reaction (PCR) and ELOSA. Colony counts were performed on bacterial suspensions and dilutions were made to the calculated colony forming units per reaction (cfu/rxn). The calculated number of bacteria (cfu?rxn) represents the lowest dilution at which sensitivity is 100% for each strain.The threshold represents the limit of detection representing two times the standard deviation above the mean of the negative controls (non-*Cms* DNA). (A) represents the DNA amplified utilizing primer set Cms50 (forward and reverse) and the corresponding hybridization probe Cp50. (B) represents the DNA amplified from each *Cms* strain utilizing primer set Cms72 and probe Cp72 used to hybridize the PCR product for the plate capture assay.

TABLE 2—*PCR/Gel electrophoresis results by* Cms *strain.*

Primer	Cfu/rxn (approx.)	As-1	OFF	Wi-2	SD-1	INM-1
Cms50	240	+/-	+	+/-	+	-
	24	+	-	+/-	-	+
	2.4	-	-	-	+	-
	0.4	-	-	-	-	-
endpoint	"0"	-	-	-	+/-	-
Cms72	240	+/-	+	+	+/-	-
	24	+/-	+	+	+/-	+
	2.4	-	+	+	-	+
	0.4	-	+	+	+/-	+
endpoint	"0"	-	+/-	-	-	-

(+) = band corresponding to the expected bp size was visualized by ethidium bromide staining.
(+/-) = band was barely visualized.
(-) = no band was visualized.
Bacterial suspensions evaluated by PCR/gel electrophoresis in the shaded areas correspond to the ELOSA results presented in Figure 1.
"No DNA" controls included in the study never produced amplified product.

nonmucoid strains was not statistically significant (Table 3). No significant difference was seen between blender and soak sample preparation, dilutions, primers, or among cultivars.

The effects of tuber-processing methods on PCR sensitivity were evaluated in the sample preparation subset. Population values of the macerated, undiluted samples from the single *Cms*-infected tubers ranged from 7.7×10^4 to 3.6×10^6 IFU/g. These values correspond to IFAS values of 0.3 to 4.6 IFU/hpf. The average soak and blender subsample ranged from 1×10^4 to 1×10^5 IFU/g. Sensitivity in the sample preparation subset was significantly different (Pr>F = 0.05) for sample preparation (Pr>F = 0.0001), primer (Pr>F = 0.0001), cultivar (Pr>F = 0.0001), and PCR product evaluation method (Pr>F = 0.0001). No significant differences were observed among strains or dilutions (Table 4).

Infected tuber tissue processed by macerating was detected by PCR with 85% sensitivity, significantly more sensitive than soak fluid (71%) and blender filtrate (63%) (Table 4). Sensitivity was significantly less for cores processed with 1:200 uninfected cores than for the 1:100 composite. PCR with primer Cms72 was a significantly more sensitive test than the PCR using Cms50 (Table 4). As in the strain x cultivar experiment, amplified PCR products evaluated by ELOSA provided a significantly more sensitive test than PCR products evaluated by gel electrophoresis (Table 4). PCR/gel electrophoresis vs. PCR/ELOSA were evaluated separately, ELOSA detected *Cms* in the asymptomatic macerated samples with 95.8% sensitivity (Table 4).

DISCUSSION

PCR has become an important method of detection for a wide range of plant pathogens in various hosts (Henson and French 1993). PCR uses pathogen-specific primers for detection of target sequences in crude specimens, without culturing (Henson and French 1993). PCR detection of plant pathogenic bacteria has not been widely adopted by diagnostic laboratories, largely because of the need for laborious DNA extractions, sophisticated equipment, and the perception that the technique is not cost effective. We feel that the data presented here will aid in making PCR more useful for routine diagnostics for BRR and tuber indexing primarily because the procedure is nonra-

TABLE 3—*Mean sensitivity (%) of PCR across three cultivars, five strains, two dilutions, two tissue processing method, two primers, and two PCR evaluation methods in data set "strain x cultivar".*

		N =	PCR evaluation methods combined (% sensitivity)	Gel electrophoresis (% sensitivity)	ELOSA (% sensitivity)
Strain	As1 (mucoid)	16	76.0	72.9	79.2
	OFF (mucoid)	16	82.3	79.2	85.4
	Wi2 (intermediate)	16	82.3	77.1	87.5
	SD1 (nonmucoid)	16	81.3	81.3	81.3
	INM (nonmucoid)	16	67.7*	58.3*	77.1
	LSD ($P = 0.05$)		12.2	19.5	NS
Process	Blender fluid	40	81.7	77.5	85.8
	Soak fluid	40	74.2	70.0	78.3
			NS	NS	NS
Dilution**	10^{-1}	40	78.8	73.3	84.2
	10^{-2}	40	77.1	74.2	80.0
			NS	NS	NS
Primer	Cms 72	40	80.8	74.2	87.5
	Cms 50	40	75.0	73.3	76.7
	LSD =		NS	NS	NS
Cultivar	Red Norland	16	75.6	71.3	80.0
	Russet Burbank	16	80.0	72.5	87.5
	Norchip	16	78.1	77.5	78.8
			NS	NS	NS
PCR evaluation	Gel electrophoresis	40	73.8		
	ELOSA	40	82.1*		
	LSD ($P = 0.05$)		7.7		

Values that are significantly different ($P = 0.05$) are denoted by *
NS = not significant
**The average IFU/rxn is calculated to be 280 and 28 cells per reaction for the 10^{-1} and 10^{-2} dilutions, respectively.

dioactive and ELISA equipment is readily available in most diagnostic laboratories.

The primers used in this study were derived from subtractive hybridization fragments and were previously shown to specifically amplify DNA from all 29 strains evaluated (Mills *et al.* 1997). Subtractive hybridization allows the selection of a DNA fragment unique to *Cms* by hybridizing and removing sequences that are in common with other organisms. The aim of this study was to examine the usefulness of these primers in a PCR protocol for the detection of *Cms* in naturally infected field potatoes.

Our experiments on asymptomatic tubers provide information on the type of testing that can take place when using PCR for bulk tuber testing. Most importantly, at least one dilution of the crude sample must be made to attenuate the inhibition of the amplification process in the PCR reaction. In addition, at least two 10-fold dilutions should be amplified to ensure high sensitivity. Further refinements are needed, however, to develop a method of DNA extraction that can attenuate the inhibition in tuber samples.

The detection level for different strains of *Cms* used to inoculate tubers was found to be statistically significant; however, there was no statistical difference in detection levels among mucoid vs. nonmucoid strains of *Cms*. These results are significant when compared to the 0% sensitivity in detecting nonmucoid strains by the commercial, double antibody sandwich ELISA (Agdia) kit employing the 1H3 antibody (Baer and Gudmestad 1993).

The ELOSA was more sensitive than gel electrophoresis for evaluating PCR products. Bands that were barely visible on an agarose gel produced a definite positive result in ELOSA. The detection limit for purified digoxigenin-labeled DNA for the combined PCR products was as little as 100 pg of each captured PCR product by ELOSA (B. Russell, personal communication).

The detection sensitivity for *Cms* in potato extracts by IFAS or any other detection assay has been estimated to no less than 10^4 cfu/ml (Baer and Gudmestad 1993, Schaad *et al.* 1999). In addition, the detection level for *Cms* by ELISA is strain dependent; nonmucoid strains cannot be detected by ELISA. This study demonstrates that PCR with the Cms50 and Cms72 primers can detect ≤3 cfu/reaction. The use of labeled probes in a plate capture assay can increase PCR sensitivity another 100-fold (Roche, PCR ELISA product insert).

The method of tuber processing was chosen to reflect the official procedure for the BRR-ELISA protocol used for Phytosanitary Certification/Export to Canada. The protocol requires that 400 tubers be tested in two, 200-tuber bulk subsamples (NDSU Cooperative Extension, Plant Pest Diagnostic Laboratory, Fargo, ND). The Canada Seed Potato Certification Program required sample size for BRR testing was adjusted in 1999 to lot size: 1% of tubers (minimum of 5) to a maximum of 400 (Canadian Food Inspection Agency, Plant Products Directorate, Plant Health And Production Division, Nepean, Ontario, Canada, D-98-01 March 17, 1999 [1st Revision] *Import requirements for seed potatoes and other potato propagative material*).

We compared the recommended subsamples containing one infected tuber core in 199 uninfected cores vs subsamples that contained one infected core in half as many uninfected

TABLE 4—*Mean sensitivity (%) of PCR across five processing methods, two dilutions, three cultivars, two* Cms *strains, two primers, and two methods of PCR evaluation for "sample preparation" data subset.*

		N =	PCR evaluation methods combined (% sensitivity)	Gel electrophoresis (% sensitivity)	ELOSA (% sensitivity)
Strain	OFF	40	64.1	55.0	73.3
	INM	40	62.5	51.7	73.3
			NS	NS	NS
Process	Macerated**	16	85.4*	75.0	95.8*
	Blender fluid 1:100	16	62.5	54.2	70.8
	Blender fluid 1:200	16	50.0*	41.7	58.3
	Soak fluid 1:100	16	70.8	62.5	79.2*
	Soak fluid 1:200	16	47.9*	33.3	62.5
	LSD (P=0.05)		17.8	24.9	26.8
Dilution***	10^{-1}	40	64.1	53.3	75.0
	10^{-2}	40	62.5	53.3	71.7
			NS	NS	NS
Primer	Cms 72	40	70.0*	66.7*	73.3
	Cms 50	40	56.7	40.0	73.3
	LSD (P=0.05)		11.3	15.8	NS
Cultivar	Red Norland	40	71.3	60.0	82.5
	Russet Burbank	40	63.7	55.0	72.5
	Norchip	40	55.0*	45.0	65.0
	LSD (P=0.05)		13.6	NS	NS
PCR evaluation	Gel electrophoresis	40	53.3		
	ELOSA	40	73.3*		
	LSD (P=0.05)		11.3		

Values that are significantly different (P = 0.05) are denoted by *
NS = not significant
**The average IFU/rxn is calculated to be 50 IFU per reaction in the macerated samples.
***Subsamples bulked 1:100 contain approximately 5 and 2.5 IFU/rxn in the 10^{-1} and 10^{-2} dilutions, respectively. Subsamples bulked 1:200 contain approximately 3 and 1 IFU/rxn in the 10^{-1} and 10^{-2} dilutions, respectively.

cores (1:100). When PCR evaluation methods were analyzed together, tuber core samples at a ratio of 1 : 200 (infected : healthy) were detected with significantly less sensitivity than at a ratio of 1 : 100. This suggests that to ensure a true positive detection, a 100 tuber subsample is more reliable, with single-tuber testing being the ideal situation. An obvious drawback of this is that single tuber processing is unrealistic for post-harvest screening.

We also compared the methods of subsample processing (soak vs blender), where the soak method is thought to increase quantities of antigen for ELISA testing. We found no difference in sensitivity detection for composite samples soaked overnight or processed immediately by the blender method. These results are consistent with the findings of other investigators (Schaad *et al.* 1999)

In our sample preparation study, the naturally infected tubers had been chosen for their low *Cms* populations by IFAS, with IFU/hpf values considered negative or questionable results, in most cases, if reported by a diagnostic laboratory. According to the BRR protocol, A.P.H.D., "Counts of ...≥5cell/hpf at the 1/10, 1/50, or 1/100 dilution in a slide window at 1000X magnification are considered positive" (Boucher 1995:33). The ability of the PCR/ELOSA to detect the macerated samples at 95.8% sensitivity when the IFAS results ranged in the +/- diagnostic range clearly shows the usefulness of this test (Table 4). Taken into consideration that ELISA currently used in seed certification would not detect nonmucoid *Cms* strain INM in the sample preparation data subset, PCR is a definite improvement.

Advantages of PCR/ELOSA over classical PCR include (a) simplicity of the standard ELISA procedure, (b) ELOSA can be used in large scale, (c) import requirements currently in use for seed testing by ELISA can eventually be adapted to the DIG-labeled PCR/ELOSA. A disadvantage we observed was that undiluted potato tissue sample, from soak or blender methods of preparation, often failed to amplify. DNA polymerase inhibition is a well-recognized problem for PCR techniques that use DNA amplification, inhibition of PCR by plant samples has been reported by others (Schaad *et al.* 1999). To circumvent the problem, 10-fold dilutions of the tissue sample were performed.

The ability to automate PCR procedures is considered a key factor in determining the role of PCR in the average laboratory. As a high-throughput alternative, the PCR/ELOSA method utilizing two unique *Cms* DNA sequence regions identified by Mills *et al.* (1997) has improved reliability of identifying *Cms* over classical PCR and ELISA.

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