A Real-Time PCR Assay for the Detection of *Clavibacter michiganensis* subsp. *sepedonicus* Based on the Cellulase A Gene Sequence

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ABSTRACT

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Clavibacter michiganensis subsp. *sepedonicus*, causal agent of bacterial ring rot (BRR) of potato (*Solanum tuberosum*), is a globally important quarantine pathogen that is managed in North America using zero tolerance regulations in the certified seed industry. *C. michiganensis* subsp. *sepedonicus* is well documented to cause symptomless infections in potato, contributing to its persistence in certified seed stocks. Reliable laboratory methods to detect symptomless infections with a high degree of sensitivity could assist in the reduction of inoculum in certified seed potato stocks. A real-time polymerase chain reaction (PCR) assay was developed using the cellulase A (CelA) gene sequence as the basis for primer design. CelA primers were specific to *C. michiganensis* subsp. *sepedonicus* grown in vitro and did not detect any other coryneform bacteria or potato pathogenic bacteria but did detect 69 strains of *C. michiganensis* subsp. *sepedonicus*. The CelA real-time PCR assay was more sensitive than immunofluorescence (IFA) and Cms50/72a PCR assays in detecting *C. michiganensis* subsp. *sepedonicus* in infected potato tuber cores blended with healthy tuber cores in simulated seed lot contamination experiments. CelA primers detected nonmucoid and mucoid strains with equivalent sensitivity. In naturally infected seed lots, CelA PCR primers also were more sensitive in detecting symptomless infections of *C. michiganensis* subsp. *sepedonicus* in seed tubers prior to planting compared to Cms50/72a PCR primers, IFA, and enzyme-linked immunosorbent assay. A real-time PCR format using the newly developed CelA primers proved to be a very robust detection tool for *C. michiganensis* subsp. *sepedonicus* with the added advantage of detecting only virulent strains of the ring rot bacterium.

Bacterial ring rot (BRR), caused by the gram-positive bacterium *Clavibacter michiganensis* subsp. *sepedonicus*, remains a threat to the potato (*Solanum tuberosum*) industry in North America. Although the disease occurs sporadically, serious outbreaks of BRR have occurred in a number of potato producing areas over the past several years in the United States and Canada. One of the key tactics for management of BRR is the use of certified seed that has been visually inspected for the disease. BRR is a highly regulated "zero tolerance" disease in the production of certified seed. No level of the disease is tolerated, and a certified seed field or seed lot is rejected if any plant or plant tissue infected with BRR is found during the inspection process.

Unfortunately, zero tolerance for BRR alone has been insufficient for long-term management of the disease since ring rot infections can remain symptomless or latent (8,33,43). A number of factors have been associated with the lack of BRR disease expression in potato, including the potato cultivar, strain of the bacterium, initial inoculum dose, and environmental conditions (8,33,43,44). Additionally, *C. michiganensis* subsp. *sepedonicus* can persist for extended periods of time on contaminated potato production surfaces (42), allowing low levels of the bacterium to exist, and thereby increasing the potential of the pathogen to infect disease-free seed stocks. BRR symptom expression can vary from season to season among potato cultivars, and a number of cultivars have been identified in which visual inspections are inadequate for detection (14,27,34,35). For this reason, postharvest testing has been implemented in several areas in an effort to reduce the risk of seedborne infections of *C. michiganensis* subsp. *sepedonicus*.

A number of tests have been developed to detect *C. michiganensis* subsp. *sepedonicus* with high sensitivity and specificity. The first tests developed were serologically based, with *C. michiganensis* subsp. *sepedonicus*–specific monoclonal antibodies utilized in immunofluorescent-antibody staining (IFA) (17) and enzyme-linked immunosorbent assays (ELISA) (18). Serological methods still are being refined using monoclonal antibodies developed against the ring rot pathogen (47). However, ELISA has been demonstrated to be ineffective in the detection of nonmucoid or nonfluidal strains of *C. michiganensis* subsp. *sepedonicus* (3). This phenomenon is thought to be due to the differences that exist in the exopolysaccharide between nonmucoid and mucoid strains of the pathogen (24). Although the impact of nonmucoid/nonfluidal strains on the global persistence of BRR in potato production is debatable, the development of nonserological methods for ring rot detection would be a critical step in the successful management of BRR.

More recently, DNA-based detection technology has been employed using a variety of methods including hybridization (20,26,50), immunocapture (5,36), and polymerase chain reaction (PCR) (29,31, 37,48,50,52,53). PCR is becoming more commonly used for detection of *C. michiganensis* subsp. *sepedonicus,* and a wide array of DNA and RNA primers have been developed specifically to detect it (6,29,31, 37,38,48,50,52). Although most *C. michiganensis* subsp. *sepedonicus*–specific primers were developed for a classical PCR format, some of these primers have been used successfully in a real-time format (2,6,52,55). The three *C. michiganensis* subsp. *sepedonicus*–specific primer sets developed by Mills et al. (37), Cms50, Cms72, and Cms85, are perhaps the most commonly used in conventional PCR. However, of these three primer sets, Cms85 has been demonstrated to be less reliable and sensitive and is used less frequently than the other two primer sets (5).

PCR-based detection methods that target virulence genes have been useful in distinguishing virulent from avirulent forms of *Streptomyces scabies* (56). The mechanism of pathogenesis for *C. michiganensis* subsp. *sepedonicus* was initially thought to be the exopolysaccharide (EPS) that plugged the xylem tissue of potato plants (57); however, this was not universally accepted since nonmucoid and mucoid strains with equal virulence had EPS with substantially different biochemical characteristics (24). The involvement of a cellulase enzyme as a primary virulence determinant was suggested because of in vitro production (4) and electron microscopic studies that revealed large sections of xylem tissue that had been compromised enzymatically (N. C. Gudmestad, *unpublished*). Subsequent to the aforementioned

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studies, it was demonstrated that a cellulase enzyme produced by *C. michiganensis* subsp. *sepedonicus* was responsible for virulence of the pathogen in planta (45). The cellulase enzyme is encoded on the native plasmid (22,28) described and designated pCS1 by Mogen and Oleson (40). The objective of the studies reported here was to develop *C. michiganensis* subsp. *sepedonicus*–specific primers based on the cellulase A gene sequence to be used in a real-time PCR.

MATERIALS AND METHODS

PCR assays. The first set of primers used in these studies to detect *C. michiganensis* subsp. *sepedonicus* is based on the Cms50 and Cms72 fragments (GenBank accession nos. AF001266 and AF001267, respectively) designed using subtraction hybridization of its genome (37). The forward and reverse primer sequences for Cms72 were modified from the original primer sequence for our real-time PCR format to facilitate binding of the probe and were designated Cms72a (Table 1). Cms72 yields an amplicon size of 164 bp, while primer sets Cms50/72a amplify single-copy *C. michiganensis* subsp. *sepedonicus*–specific DNA fragments of 192 and 213 bp, respectively. For classical multiplex PCR with primers Cms50/72a, 0.25 μM of each forward and reverse primer were added to the mix consisting of 1× polymerase buffer, 2 mM $MgCl_2$, 0.5 mM dNTP (Promega), 1.5 units of Amplitaq Gold polymerase (Applied Biosystems), and 2 μl of template DNA in a 25-μl reaction. The cycling conditions involved initial incubation for 5 min at 95°C, followed by 10 cycles of 45 s at 94°C, 1 min at 60°C, and 15 s at 72°C, another 40 cycles of 45 s at 92°C, 40 s at 60°C, and 20 s at 72°C, and finally an extension at 72°C for 10 min. The amplification with primer CelA was performed by adding 0.5 μM of each forward and reverse primer to the mix consisting of 1× polymerase buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1 unit of Amplitaq Gold polymerase, and 2 μl of template DNA in a 25-μl reaction. The cycling conditions involved initial incubation for 5 min at 95°C, followed by 40 cycles of 30 s at 92°C, 45 s at 60°C, and 30 s at 72°C, and finally an extension at 72°C for 7 min.

The fluorogenic probe specific for Cms50 was labeled with reporter dye 6-carboxy-fluorescein (FAM) (517 nm maximum emission) at the 5′ end, and the 3′ end was modified with the quencher dye BH1 (Table 1). Similarly, the probe specific for Cms72a was labeled with reporter dye Cy5 (667 nm maximum emission) at the 5′ end, and the 3′ end was modified with the quencher dye BH2 (Sigma Genosys) (Table 1). Real-time (TaqMan) PCR was performed in 0.2 ml optical tube strips (Stratagene) using Stratagene Mx3005P QPCR system. The real-time PCR assay was performed in a 25-μl reaction consisting of 4 μl of template DNA, 1× FastStart Universal Probe Master, Rox (Roche), Cms50F and Cms50R primers at a concentration of 0.4 μM, Cms72aF and Cms72aR primers at 1 μM, and the corresponding probes at 0.4 μM and 0.6 μM, respectively. PCR grade water was added to a final volume of 25 μl. A two-step cycling protocol was used with an initial incubation of the mix at 95°C for 10 min followed by 40 cycles at 95°C for 30 s, 60°C for 45 s, and 72°C for 30 s with data capture at the end of each 72°C incubation.

The second primer set, CelA-F and CelA-R, was designed based on the cellulase A gene sequence encoded by the native plasmid pCS1 of *C. michiganensis* subsp. *sepedonicus* (GenBank accession no. AY007311). The primers amplify a 150-bp region in the cellulase A gene sequence (Table 1). Transcription of the cellulase A gene is initiated at nucleotide 568, with the promoter region running from nucleotide 566 to 575 (28). The CelA primer set amplifies from region 598 to 747. The fluorogenic probe specific for CelA was labeled with reporter dye Hex (553 nm maximum emission) at the 5′ end, and the 3′ end was modified with the quencher dye BH2 (Table 1). Real-time PCR was performed in a 25-μl reaction consisting of 2 μl of template DNA, 0.8× FastStart Universal Probe Master, Rox (Roche), CelA-F and CelA-R primers at a concentration of 0.75 μM, and the probe at a concentration of 0.16 μM. PCR grade water was added to a final volume of 25 μl. The real-time PCR protocol was identical to that described for Cms50/72a primer sets. A positive crossing threshold (Ct) of 35 cycles was used in all experiments for all *C. michiganensis* subsp. *sepedonicus* primer sets to determine the presence of the organism. The MX4000v 1.00 to 3.00 algorithm software in Stratagene MX3005P was utilized to automatically calculate and adjust the baseline Ct value. Additionally, the master mixture for real-time PCR containing ROX (Roche Applied Sciences) was included to serve as internal control for data normalization.

Specificity of the CelA real-time PCR assay. The specificity of the CelA primers was determined initially using bacteria grown in vitro added to the PCR mix directly, with no DNA extraction procedure utilized by testing 69 strains of *C. michiganensis* subsp. *sepedonicus* randomly selected from our extensive culture collection (Table 2). The specificity of the CelA primers was determined also by testing non–*C. michiganensis* subsp. *sepedonicus* bacterial strains from our collection, including *Clavibacter rathayi*, *C. tritici*, *Leifsonia xyli* subsp. *cynodontis*, *Corynebacterium matruchotti*, *Curtobacterium flaccufaciens*, *Pectobacterium carotovora* subsp. *atroseptica*, *Rhodococcus fascians,* and *Streptomyces scabies* (Table 3). Additional specificity evaluations included the following subspecies of *C. michiganensis*: *C. michiganensis* subsp. *insidiosus*, *C. michiganensis* subsp. *michiganensis, C. michiganensis* subsp. *nebraskensis,* and *C. michiganensis* subsp. *tessellarius* (Table 3). All specificity experiments were performed twice.

Sensitivity of Cms50/72a and CelA PCR using in vitro *C. michiganensis* subsp. *sepedonicus*. The sensitivity of CelA and Cms50/72a primers were compared using classical and real-time PCR assays. Bacterial strain OFF-1 was cultured in liquid nutrient broth yeast extract medium (NBY) for 24 to 48 h at room temperature and adjusted to an optical density of 0.1 at 560 nm, corresponding to approximately 1×10^8 cells/ml, and subsequently serially diluted 10-fold using molecular biology grade water. In addition to this, total genomic DNA was extracted from a 48-h culture of bacterial strain OFF-1 according to manufacturer's specifications (Wizard Genomic DNA purification kit, Promega). The final DNA concentration was adjusted to 10 ng/μl and then serially diluted 10-fold with water. Both bacterial cells and purified DNA were tested using CelA and Cms50/72a PCR assays performed as previously described to determine if any reduction in sensitivity occurs when DNA is not extracted. All sensitivity experiments were performed twice.

Sensitivity of CelA real-time PCR using controlled *C. michiganensis* subsp. *sepedonicus* tuber infections. Several experiments were conducted to compare diagnostic methods for detecting *C. michiganensis* subsp. *sepedonicus* in simulated BRR-infected seed lots. The objective of

Table 1. Real-time polymerase chain reaction oligonucleotide primer and fluorescent probes sequences for Cms50, Cms72a, and CelA

Primer/probe name	Sequence (5′-3′)
Cms50F	GAGCGCGATAGAAGAGGAACTC
Cms50R	CCTGAGCAACGACAAGAAAAATATG
Cms72aF	CTACTTTCGCGGTAAGCAGTT
Cms72aR	GCAAGAATTTCGCTGCTATCC
Cms50 probe	[DFAM] TGAAGATGCGACATGGCTCCTCGGT [DBH1]
Cms72a probe	[DCY5] GATCGTGAATCCGAGACACGGTGACC [DBH2]
CelA-F	TCTCTCAGTCATTGTAAGATGAT
CelA-R	ATTCGACCGCTCTCAAA
CelA probe	[DHEX] TTCGGGCTTCAGGAGTGCGTGT [DBH2]

these experiments was to artificially produce asymptomatic but *C. michiganensis* subsp. *sepedonicus*–infected seed lots. Infected tubers were produced by inoculating seed pieces freshly cut from certified seed-tubers cv. Russet Burbank. Seed pieces were vacuum infiltrated with ¼ strength nutrient broth containing either mucoid or nonmucoid *C. michiganensis* subsp. *sepedonicus* suspension of 10^8 or 10^4 cells/ml. Tubers were placed into a desiccator, broth was added to ensure complete coverage of all seed pieces, and a vacuum was applied and maintained at 103 kPa in the desiccator for 15 min. Tubers subsequently were removed and allowed to dry on the bench top overnight. Twenty-five seed pieces were planted in 9-m rows in late May in 2005, 2006, and 2007. Fertilizer, insecticides, and fungicides were applied as necessary throughout the growing season. Tubers were harvested in early October in all 3 years, 125 to 130 days after planting. Approximately 20 to 30 asymptomatic tubers were selected from plants with foliar symptoms of BRR for use in evaluating existing and experimental diagnostic assays.

From each infected tuber selected, as well as each infection-free tuber, an approximately 1-g core was excised from the stem end of the tuber using a sterilized common apple corer as previously described (19). One infected tuber core was combined with infection-free tuber cores, diluted 1:2 in sterile water, and shaken for 16 to 20 h. Aliquots collected from the resulting soak solutions were used in analyses to mimic as closely as possible standard protocols followed by certification agencies in which 200 tuber core lots are processed. In the initial preliminary trial performed in 2005, asymptomatic but infected tubers, as confirmed by IFA described below, were blended with healthy tubers in ratios from one infected tuber per 199 healthy tubers (1/200) to 10 infected tubers blended with 190 healthy tubers (10/200). In subsequent experiments, *C. michiganensis* subsp. *sepedonicus*–infected tubers with several population density levels were blended with healthy tubers in ratios of 1/100, 1/200, and 1/400. In all experiments, the soak liquid from *C. michiganensis* subsp. *sepedonicus*–free cores was used as negative controls for all assays as well as to make the dilutions from 1:100 to 1:200 and 1:400, as each infected core could only be soaked once. The PCR was performed on various dilutions of tuber soak fluids to rule out the possibility of PCR inhibition due to compounds originating from host tissue. All IFA, ELISA, and PCR assays were performed twice.

The *C. michiganensis* subsp. *sepedonicus* population density in each asymptomatic tuber was determined using IFA before being used to blend with healthy potato tubers as previously described (3).

Table 2. *Clavibacter michiganensis* subsp. *sepedonicus* isolate designation and real-time polymerase chain reaction with primers Cms50/72a and CelA

Isolate designation	Geographic origin	Source	Cms50/72a	CelA	pCS1[a]
5	Unknown	NDSU collection	+	+	nd
001	South Dakota	NDSU collection	+	+	nd
002, Wi2	Wisconsin	NDSU collection	+	+	nd
002l, Wi2	Wisconsin	NDSU collection	+	+	nd
002s, Wi2	Wisconsin	NDSU collection	+	+	nd
003	South Dakota	NDSU collection	+	+	nd
004	South Dakota	NDSU collection	+	+	nd
005	South Dakota	NDSU collection	+	+	nd
076-10	Minnesota	NDSU collection	+	+	nd
075-14	Wisconsin	NDSU collection	+	+	nd
084-3	Idaho	NDSU collection	+	+	nd
087-2	Idaho	NDSU collection	+	+	nd
087-24	Idaho	NDSU collection	+	+	nd
096A	North Dakota	NDSU collection	+	+	nd
097A	North Dakota	NDSU collection	+	+	nd
099A	North Dakota	NDSU collection	+	+	nd
100A	North Dakota	NDSU collection	+	+	nd
102-1	Idaho	NDSU collection	+	+	nd
103	North Dakota	NDSU collection	+	+	nd
104	North Dakota	NDSU collection	+	+	nd
116-1-1	Idaho	NDSU collection	+	+	nd
116-6-5	Idaho	NDSU collection	+	+	nd
116-11-3	Idaho	NDSU collection	+	+	nd
116-16-1	Idaho	NDSU collection	+	+	nd
116-19-3	Idaho	NDSU collection	+	+	nd
116-24-3	Idaho	NDSU collection	+	+	nd
117-6	Washington	NDSU collection	+	+	nd
128	North Dakota	NDSU collection	+	+	nd
135	North Dakota	NDSU collection	+	+	nd
136	North Dakota	NDSU collection	+	+	nd
137	North Dakota	NDSU collection	+	+	nd
141	Unknown	NDSU collection	+	+	nd
151-3	North Dakota	NDSU collection	+	+	nd
192 1.3	North Dakota	NDSU collection	+	+	nd
AS-1	Minnesota	NDSU collection	+	+	nd
AS-1-R	Minnesota	NDSU collection	+	+	nd
ATCC9850	New York	ATCC	+	+	E
ATCC33113	Canada	ATCC	+	+	E
BETA1230	North Dakota	NDSU collection	+	+	nd
BJ19	Oregon	NDSU collection	+	+	nd
BRR7	Brit Columbia	S. De Boer	+	+	E
COL 1-B	North Dakota	NDSU collection	+	+	nd
COLO 18	Colorado	NDSU collection	+	+	nd
CS3M	Alberta	S. De Boer	+	+	E
CS3NM	Alberta	S. De Boer	+	+	E
CS3R	Alberta	S. De Boer	+	+	E
CS3RC	Alberta	S. De Boer	+	+	nd
CS5	New York	S. De Boer	+	+	E
CS14 (14)	Montana	S. Slack	+	-	-
CS16	New Brunswick	S. De Boer	+	+	E
CS17	Maine	S. De Boer	+	+	E
CS20	Wisconsin	S. Slack	+	+	I
CS106	W. Virginia	S. De Boer	+	+	I
CSCA	California	A. Vidaver	+	+	E
CSIDNM1	Idaho	D. Clarke	+	+	E
CSIDNM2	Idaho	D. Clarke	+	+	nd
CSMT	Montana	A. Vidaver	+	+	E
CSSS-43	Wisconsin	S. Slack	+	+	E
CSSS-44	Wisconsin	S. Slack	+	+	E
INM-1	Idaho	D. Clarke	+	+	nd
NZ2531	Sweden	PDDCC	+	+	E
NZ2532	Canada	PDDCC	+	+	E
NZ2534	New York	PDDCC	+	+	E
NZ2535	Canada	PDDCC	+	+	I
NZ2537	U.S.A.	PDDCC	+	+	E
OFF	North Dakota	NDSU collection	+	+	nd
P45	Unknown	S. De Boer	+	-	-
R1	Brit Columbia	S. De Boer	+	+	I
R2	Brit Columbia	S. De Boer	+	+	I
R3	Brit Columbia	S. De Boer	+	+	I
R4	Brit Columbia	S. De Boer	+	+	I
R5	Brit Columbia	S. De Boer	+	+	I

[a] Presence of pCS1 plasmid is denoted as episomal (E), chromosomally integrated (I), absent (-), or was not determined (nd) (41).

Each asymptomatic and presumed infected tuber selected was aseptically cross-sectioned approximately 2.5 cm from the stem end, and a 1-g section of vascular tissue was extracted. Vascular tissue sections were diluted in 1 ml of sterile phosphate buffered saline (PBS), resulting in a 1:2 dilution, and manually crushed to release bacterial cells. Two 10-fold serial dilutions were performed subsequently in PBS and added to individual wells of an 18-well microscope slide. Each well was stained using monoclonal antibody 9A1 mouse anti-*C. michiganensis* subsp. *sepedonicus* (17) and goat anti-mouse IgG and IgM polyclonal fluorescent labeled antibody following methods provided by Agdia Inc. (Elkhart, IN). A minimum of 10 high-powered (×1,500) microscope fields (MF) were examined for the presence of fluorescent cells having the size and shape typical of *C. michiganensis* subsp. *sepedonicus* for each dilution well. The number of immunofluorescent units (IFU) was converted quantitatively and expressed as IFU/g tuber fresh weight (3). Vascular tissue sections were also tested using CelA PCR to confirm that cells visualized by IFA were not serological cross-reactors. Additionally, asymptomatic tubers from BRR-infected potato plants that produced a negative IFA reaction were tested with real-time PCR using Cms50/72a and CelA primers to determine if the tubers were healthy or latently infected with *C. michiganensis* subsp. *sepedonicus*. One or two tubers each of mucoid and nonmucoid, high (>10^7 IFU/g), moderate (10^6 to 10^7 IFU/g), and low (<10^5 IFU/g) infection levels were subsequently selected for use in the simulated BRR-contaminated seed lot trials. In 2007, only IFA-negative but PCR-positive tubers, indicating a symptomless infection of *C. michiganensis* subsp. *sepedonicus*, were used for further experiments to represent a low level of infection. Healthy tubers were selected from certified seed cv. Russet Burbank, processed as described above and confirmed by real-time PCR to be free from *C. michiganensis* subsp. *sepedonicus* before being blended with asymptomatic tubers or used as healthy controls.

ELISA was performed using capture by mouse monoclonal antibody 1H3 (18) in a double antibody sandwich (DAS) with detection by a polyclonal antibody labeled with alkaline phosphatase following provided protocols (Agdia). Aliquots of soak liquid representing each sublot of tuber composites were tested in triplicate wells on separate ELISA plates. Enzyme substrate was allowed to develop for 40 min before absorbance values were taken. Absorbance values (405 nm) >0.2 were considered positive for the detection of *C. michiganensis* subsp. *sepedonicus*.

Commercial seed lot testing. In addition to controlled experiments using simulated *C. michiganensis* subsp. *sepedonicus*

Table 3. Bacterial name designation and real-time polymerase chain reaction (PCR) with primers Cms50/72a and CelA

Bacterial name	Isolate designation	Geographic origin	Source	Cms50/72a[a]	CelA
Clavibacter michiganensis subsp. *insidiosus*					
	0.0239	Unknown	L. Clafin	-	-
	ATCC10253	Kansas	ATCC	-	-
	ATCC33114	Unknown	ATCC	-	-
	CI-4	Unknown	S. De Boer	-	-
	CI102B	Colorado	S. De Boer	-	-
	CIAW81-3	Colorado	S. De Boer	-	-
	CILETH	Colorado	S. De Boer	-	-
	CIN	Unknown	S. De Boer	-	-
	CIN53	Colorado	S. De Boer	-	-
	NZ2611	U.K.	PDDCC	-	-
	NZ2621	U.S.A.	PDDCC	-	-
	NZ2948	New Zealand	PDDCC	-	-
	NZ3567	Australia	PDDCC	-	-
	NZ3619	Australia	PDDCC	-	-
	NZ3983	U.S.A.	PDDCC	-	-
	NZ4191	New Zealand	PDDCC	-	-
	NZ4543	Unknown	PDDCC	-	-
	NZ6565	New Zealand	PDDCC	-	-
Clavibacter michiganensis subsp. *michiganensis*					
	0.01104	Kansas	L. Clafin	-	-
	ATCC4450	Unknown	ATCC	-	-
	ATCC7430	Unknown	ATCC	-	-
	ATCC10202	Unknown	ATCC	-	-
	ATCC14456	Italy	ATCC	-	-
	NZ0549	New Zealand	PDDCC	-	-
	NZ1436	New Zealand	PDDCC	-	-
	NZ1808	New Zealand	PDDCC	-	-
	NZ1811	New Zealand	PDDCC	-	-
	NZ2355	New Zealand	PDDCC	-	-
	NZ2539	South Africa	PDDCC	-	-
	NZ2541	U.K.	PDDCC	-	-
	NZ2545	Sicily	PDDCC	-	-
	NZ2550	Hungary	PDDCC	-	-
	NZ2551	Brazil	PDDCC	-	-
	NZ5026	U.S.A.	PDDCC	-	-
	NZ6726	New Zealand	PDDCC	-	-
Clavibacter michiganensis subsp. *nebraskensis*					
	0.01105	Colorado	L. Clafin	nd	-
	0.01106	Kansas	L. Clafin	nd	-
	0.01118	Kansas	L. Clafin	nd	-
	0.01119	Kansas	L. Clafin	nd	-
	0.01120	Kansas	L. Clafin	nd	-
	ATCC27794	Nebraska	ATCC	nd	-
	ATCC27822	Nebraska	ATCC	-	-
	CNEB	Nebraska	A. Vidaver	nd	-
	CNEB 1-1	Nebraska	A. Vidaver	-	-
	NZ3294	U.S.A.	PDDCC	nd	-
	NZ3299	U.S.A.	PDDCC	nd	-
	NZ3300	U.S.A.	PDDCC	nd	-
	NZ3303	U.S.A.	PDDCC	nd	-
	NZ5366	U.S.A.	PDDCC	-	-
	NZ5367	U.S.A.	PDDCC	-	-
	NZ5368	U.S.A.	PDDCC	-	-
	NZ5369	U.S.A.	PDDCC	-	-
Clavibacter michiganensis subsp. *tessellarius*					
	0.01122	Nebraska	L. Clafin	-	-
	NZ7220	Unknown	PDDCC	nd	-
	NZ7221	Unknown	PDDCC	nd	-
	NZ7222	Unknown	PDDCC	nd	-
	NZ7223	Unknown	PDDCC	nd	-
	NZ7224	Unknown	PDDCC	nd	-
	NZ7225	Unknown	PDDCC	nd	-
	NZ7226	Unknown	PDDCC	nd	-
	NZ7227	Unknown	PDDCC	nd	-
	NZ7228	Unknown	PDDCC	nd	-
	NZ7229	Unknown	PDDCC	nd	-

(*Continued on next page*)

[a] Real-time PCR was negative (-) or not determined (nd).

contamination rates described above, certified seed lots from North Dakota and Minnesota, known to have been previously exposed to bacterial ring rot infections, also were evaluated with all serological and PCR-based detection methods to compare detection efficiency. The certified seed lots were tested using fresh tuber material processed as previously described (19). The naturally infected seed lots were indexed in the laboratory using 2,000 to 4,400 tubers/seed lot, and all detection methods were applied to tuber cores processed into 200 tuber composites. All serological assays were performed as described above. Positive thresholds for IFA and ELISA were based on international standards (1). For IFA, a minimum of 30 MF were examined and the test was considered positive if the average IFUs (fluorescing cells with characteristic size and shape of *C. michiganensis* subsp. *sepedonicus*) were equal to or greater than 5 IFU/MF. For ELISA, absorbance values (405 nm) >0.2 were considered positive.

Seed lots tested for *C. michiganensis* subsp. *sepedonicus* were monitored the following growing season in those instances where the seed was planted commercially. Symptoms of bacterial ring rot were quantified for the incidence of ring rot by one of the authors (N. C. Gudmestad). The percentage of the disease was determined by scoring a minimum of 1,000 plants per field from 31 July to 11 August 2006. Randomly selected plants scored as being presumptively symptomatic for BRR were removed from the field and tested in the laboratory for the presence of *C. michiganensis* subsp. *sepedonicus* using CelA PCR to confirm the visual field diagnosis.

Statistical analysis. The parameter estimates of slope and intercept of the lines resulting from the regression analysis of threshold cycle and either DNA (ng/ml) or *C. michiganensis* subsp. *sepedonicus* cells/ml using Cms50 and Cms72a primers were compared to the lines resulting from CelA primers. The line resulting from evaluation using CelA primers was used as a reference for analysis.

RESULTS

CelA primers detected all strains of *C. michiganensis* subsp. *sepedonicus* in our culture collection except plasmid negative strains P45 and CS14 (Table 2) and did not detect other bacterial strains (Table 3). CelA primers did not detect *Clavibacter rathayi*, *C. tritici*, *Leifsonia xyli* subsp. *cynodontis*, *Corynebacterium matruchotti*, *Curtobacterium flaccufaciens*, *Erwinia carotovora* subsp. *atroseptica*, *Rhodococcus fascians*, *Streptomyces scabies,* or other subspecies of *Clavibacter michiganensis* such as *C. michiganensis* subsp. *insidiosus*, *C. michiganensis* subsp. *michiganensis, C. michiganensis* subsp. *nebraskensis,* and *C. michiganensis* subsp. *tessellarius.*

Table 3. (*Continued from preceding page*)

Bacterial name	Isolate designation	Geographic origin	Source	Cms50/72a[a]	CelA
Clavibacter rathayi					
	ATCC13659	U.K.	ATCC	-	-
	NZ2572	New Zealand	PDDCC	nd	-
	NZ2573	New Zealand	PDDCC	nd	-
	NZ2574	New Zealand	PDDCC	nd	-
	NZ2575	New Zealand	PDDCC	nd	-
	NZ2576	New Zealand	PDDCC	nd	-
	NZ2577	New Zealand	PDDCC	nd	-
	NZ2579	Scotland	PDDCC	nd	-
Clavibacter tritici					
	NZ2623	India	PDDCC	-	-
	NZ2624	Egypt	PDDCC	nd	-
	NZ2626	Egypt	PDDCC	nd	-
	NZ2627	India	PDDCC	nd	-
	NZ2628	Iran	PDDCC	nd	-
Clavibacter xyli subsp. *cynodontis*					
	FB-1	Unknown	M. Davis	nd	-
	TB2B	Unknown	M. Davis	-	-
Corynebacterium sp.					
	NZ6306	Australia	PDDCC	nd	-
	NZ6307	Australia	PDDCC	nd	-
	NZ6308	Australia	PDDCC	nd	-
	NZ6309	Australia	PDDCC	nd	-
	NZ6310	Australia	PDDCC	nd	-
	NZ6311	Australia	PDDCC	nd	-
	NZ6790	New Zealand	PDDCC	nd	-
	NZ6792	New Zealand	PDDCC	nd	-
	NZ6793	New Zealand	PDDCC	nd	-
Corynebacterium matruchotii					
	ATCC14264	U.S.A.	ATCC	nd	-
Curtobacterium flaccumfaciens					
	0.01102	Nebraska	M. Schuster	nd	-
	ATCC6887	Unknown	ATCC	nd	-
	CFACCV3	Unknown	J. Venette	nd	-
	CFLAC2A	Unknown	J. Venette	nd	-
Curtobacterium flaccumfaciens pv. *betae*					
	ATCC13437	Unknown	ATCC	nd	-
	NZ2591	U.K.	PDDCC	nd	-
	NZ2592	U.K.	PDDCC	nd	-
	NZ2593	U.K.	PDDCC	nd	-
	NZ2595	U.K.	PDDCC	nd	-
	NZ4735	U.K.	PDDCC	nd	-
	NZ4736	U.K.	PDDCC	nd	-
	NZ4737	U.K.	PDDCC	nd	-
	NZ7458	Brazil	PDDCC	nd	-
Curtobacterium flaccumfaciens pv. *flaccumfaciens*					
	NZ2580	U.S.A.	PDDCC	nd	-
	NZ2581	U.S.A.	PDDCC	nd	-
	NZ2582	Romania	PDDCC	nd	-
	NZ2583	Unknown	PDDCC	nd	-
	NZ2584	Hungary	PDDCC	nd	-
	NZ2585	Germany	PDDCC	nd	-
	NZ2588	U.S.A.	PDDCC	nd	-
	NZ2590	U.S.A.	PDDCC	nd	-
	NZ3495	Netherlands	PDDCC	nd	-
	NZ5370	U.S.A.	PDDCC	nd	-
	NZ5371	U.S.A.	PDDCC	nd	-
	NZ5372	U.S.A.	PDDCC	nd	-
	NZ5373	U.S.A.	PDDCC	nd	-
Curtobacterium flaccumfaciens pv. *oortii*					
	0.06-83-70-a	Netherlands	G. Secor	nd	-
	NZ2632	Netherlands	PDDCC	nd	-
	NZ3497	Netherlands	PDDCC	nd	-
	NZ3498	U.K.	PDDCC	nd	-
	NZ3499	U.K.	PDDCC	nd	-
Curtobacterium flaccumfaciens pv. *poinsettiae*					
	NZ2561	U.S.A.	PDDCC	nd	-
	NZ2563	U.S.A.	PDDCC	nd	-
	NZ2564	U.S.A.	PDDCC	nd	-

(*Continued on next page*)

The sensitivity of the CelA primers was higher in detecting *C. michiganensis* subsp. *sepedonicus* bacterial cells in classical PCR compared to Cms50/72a (Fig. 1A and C). CelA detected a population density of 10^4 cells/ml, while the Cms50/72a detection limit was 10^5 cells/ml. CelA real-time PCR was also more sensitive in detecting the cells compared to Cms50/72a (Fig. 2A). Results from regression analysis comparing real-time PCR of bacterial cells of *C. michiganensis* subsp. *sepedonicus* using Cms50, Cms72a, and CelA primers provide statistical support for the observed increased sensitivity. When compared to CelA, lines generated by Cms50 (P = 0.0004) and Cms72a (P = 0.0002) were significantly different than the line generated by CelA primers (Fig. 2A). These differences were due to an increase in both the y-intercept (Cms50 P = 0.0004, Cms72a $P < 0.0001$) and the slope (Cms50 P = 0.0126, Cms72a P = 0.0004) of the lines (Fig. 2B). The CelA and Cms72a primer did not differ in sensitivity when comparing purified DNA of plasmid positive *C. michiganensis* subsp. *sepedonicus* strain OFF-1 in either classical PCR (Fig. 1B and D) or real-time PCR (Fig. 2B). The line generated by Cms72a for bacterial cells was not significantly different than that generated by CelA (P = 0.5823). The regression line resulting from Cms50 was significantly different than CelA (P = 0.0008), both the slope (P = 0.0048) and intercept (P = 0.0002) contributed to this difference. Four 150-bp amplicons resulting from amplification using CelA primers were cloned into pGEMT Easy (Promega) and sequenced for identity confirmation. These amplicons displayed 100% homology among them as well as with the published *C. michiganensis* subsp. *sepedonicus* cellulase A gene sequence (AY007311.1) from GenBank.

In real-time PCR assays, CelA primers were more sensitive than other detection methods in detecting *C. michiganensis* subsp. *sepedonicus*–infected cores that were blended with healthy tuber cores in preliminary experiments using simulated contamination rates. The CelA primers were capable of detecting two infected cores blended with 198 healthy cores, while the limit of detection in these experiments for IFA and Cms50/72a primers was 10 infected cores in 190 healthy.

Table 3. (*Continued from preceding page*)

Bacterial name	Isolate designation	Geographic origin	Source	Cms50/72a[a]	CelA
	NZ2565	U.S.A.	PDDCC	nd	-
	NZ2567	U.S.A.	PDDCC	nd	-
	NZ2568	U.S.A.	PDDCC	nd	-
	NZ2569	U.S.A.	PDDCC	nd	-
	NZ2570	U.S.A.	PDDCC	nd	-
	NZ3500	U.S.A.	PDDCC	nd	-
Rhodococcus fascians					
	NZ2596	U.K.	PDDCC	nd	-
	NZ2597	U.K.	PDDCC	nd	-
	NZ2598	U.K.	PDDCC	nd	-
	NZ2599	U.K.	PDDCC	nd	-
	NZ2600	U.K.	PDDCC	nd	-
	NZ2601	U.K.	PDDCC	nd	-
	NZ2602	U.S.A.	PDDCC	nd	-
	NZ2603	Canada	PDDCC	nd	-
	NZ2604	U.S.A.	PDDCC	nd	-
	NZ2605	U.S.A.	PDDCC	nd	-
	NZ5339	U.K.	PDDCC	nd	-
	NZ5340	U.K.	PDDCC	nd	-
	NZ5833	U.S.A.	PDDCC	nd	-
	NZ6788	New Zealand	PDDCC	nd	-
	NZ6792	New Zealand	PDDCC	nd	-
	NZ7109	Netherlands	PDDCC	nd	-
	NZ7113	Netherlands	PDDCC	nd	-
	NZ7261	New Zealand	PDDCC	nd	-
	NZ7364	New Zealand	PDDCC	nd	-
Streptomyces scabies					
	ATCC49173	New York	ATCC	nd	-
	Car 8	Unknown	R. Loria	nd	-
Pectobacterium carotovora subsp. *carotovora*					
	028	North Dakota	NDSU	nd	-
Pectobacterium carotovora subsp. *atroseptica*					
	ECA-6	North Dakota	NDSU	-	-
	ECA-13	North Dakota	NDSU	nd	-
Staphylococcus aureus					
	84.104	Unknown	Unknown	nd	-
Escherichia coli					
	ECOLI	Unknown	Unknown	-	-

The real-time PCR assay using the CelA primers was also more sensitive than Cms50/72a primers in detecting *C. michiganensis* subsp. *sepedonicus* in simulated ring rot infected seed lot experiments conducted in 2006, regardless of the initial population density or colony type of the pathogen (Table 4). When one infected tuber core was blended with 99 healthy tuber cores (1/100), CelA successfully detected the pathogen 75.0% of the time, while Cms50/72a was effective only 37.5% of the time. When the infection rates were 1/200 and 1/400, the efficiency of CelA was 37.5 and 62.5%, respectively, while Cms50/72a was 12.5 and 0.0%, respectively (Table 4). Mucoid and nonmucoid type strains were detected with equivalent sensitivity by both CelA and Cms50/72a PCR assays.

Similar results were obtained during simulated BRR infected seed lot experiments conducted in 2007 (Table 5). CelA and Cms50/72a real-time assays were similar in the sensitivity of *C. michiganensis* subsp. *sepedonicus* detection when 1 asymptomatic tuber core was blended with 99 healthy tuber cores, with detection levels of 90.9 and 100%, respectively. However, when asymptomatic to healthy tuber core ratios were 1/200 and 1/400, CelA successfully detected infections 90.9 and 81.8% of the time, respectively, compared to Cms50/72a, in which only 63.6 and 18.2% of the infections were detected, respectively (Table 5). Again, both primer sets detected mucoid and nonmucoid infections with similar sensitivities. Interestingly, in this experiment, ELISA proved to be more sensitive in detecting asymptomatic infections of the mucoid strain OFF-1 compared to IFA. However, ELISA was ineffective in detecting the nonmucoid strain. ELISA was as sensitive as Cms50/72a real-time PCR in detecting asymptomatic mucoid infections at ratios of 1/200 and 1/400. In all simulated seed lot experiments, infection-free cores used as negative controls as well as for diluents of contaminated ratios tested negative by all applicable assays.

In naturally infected seed lots, the CelA real-time PCR assay was also more sensitive than other diagnostic methods in detecting symptomless infections of *C. michiganensis* subsp. *sepedonicus* in seed tubers prior to planting (Table 6). Among the seed lots tested, ELISA was ineffective in detecting *C. michiganensis* subsp. *sepedonicus* despite ring rot disease levels of >4% (Table 6). ELISA only detected the organism in 4 of the 11 ring rot infected certified seed lots, while IFA detected symptomless infections in 7 of 11 seed lots. Real-time PCR using the Cms50/72a and CelA primers detected the ring rot bacterium in 10 of 11 seed lots (Table 6). However, the number of composite samples found to be infected using CelA PCR was proportionately higher than for any

other test performed in several seed lots with BRR disease development >4%. Given the extremely high levels of ring rot observed in the field, with BRR infection levels approaching 20%, CelA was more efficient in detecting symptomless infections. A single false negative resulted with all detection assays in the seed lot with approximately 0.1% BRR in the field (Table 6).

DISCUSSION

The importance of *C. michiganensis* subsp. *sepedonicus* as a potato pathogen and as a quarantine pest is clearly demonstrated by the volume of research that has been dedicated toward its identification, differentiation, and detection using serological and molecular techniques (2,3,5, 6,9,15–18,20,21,23,25,26,29–32,36–39,46, 48–52,54). Not only is this body of research impressive by its sheer numbers, it also bridges three decades of work, so it can be assumed that BRR continues to be a globally important pest requiring considerable resources and continuing attention. Much of the reason for this lies in the peculiarities of the disease and the pathogen. BRR is known to remain latent, or symptomless, for long periods of time, thereby permitting the pathogen to go undetected

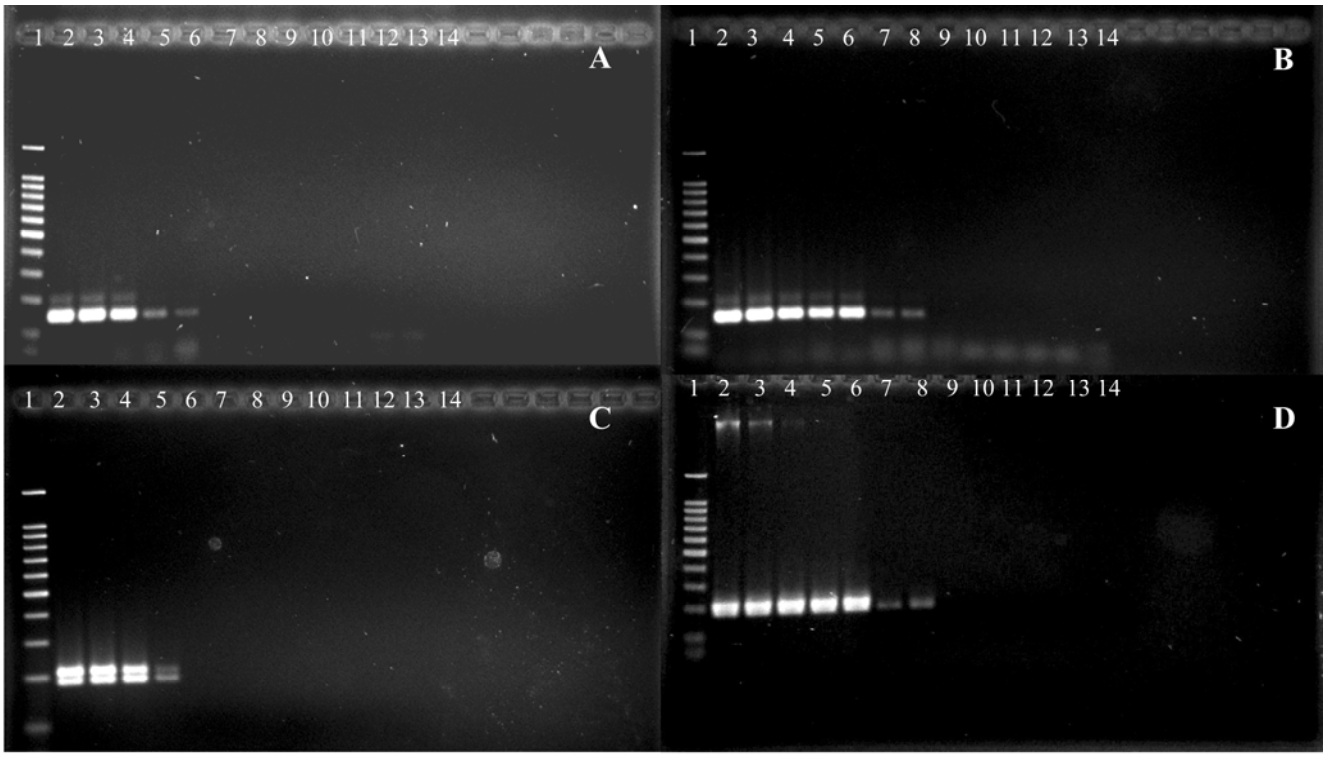

Fig. 1. A, CelA (150 bp) and **C,** Cms50/72a (double amplicons of 192/213 bp) polymerase chain reaction (PCR) amplicons of serially diluted *Clavibacter michiganensis* subsp. *sepedonicus* cells of plasmid positive strain OFF-1 electrophoresed on a 3% agarose gel. Lane 1, 100-bp Bench top ladder (Promega); lane 2, 1×10^8 cells/ml; lane 3, 1×10^7 cells/ml; lane 4, 1×10^6 cells/ml; lane 5, 1×10^5 cells/ml; lane 6, 1×10^4 cells/ml; lane 7, 1×10^3 cells/ml; lane 8, 100 cells/ml; lane 9, 10 cells/ml; lane 10, 1.0 cell/ml; lane 11, *Pectobacterium carotovora* cells; lane 12, water blank. **B,** CelA (150 bp) and **D,** Cms50/72a (double amplicons of 192/213 bp) PCR amplicons of serially diluted *C. michiganensis* subsp. *sepedonicus* strain OFF-1 DNA electrophoresed on a 2 or 3% agarose gel. Lane 1, 100-bp Bench top ladder; lane 2, 38 ng DNA/µl; lane 3, 10 ng DNA/µl; lane 4, 1.0 ng DNA/µl; lane 5, 0.1 ng DNA/µl; lane 6, 0.01 ng DNA/µl; lane 7, 1.0×10^{-3} ng DNA/µl; lane 8, 1.0×10^{-4} ng DNA/µl; lane 9, 1.0×10^{-5} ng DNA/µl; lane 10, 1.0×10^{-6} ng DNA/µl; lane 11, 1.0×10^{-7} ng DNA/µl; lane 12, 1.0×10^{-8} ng DNA/µl; lane 13, *Pectobacterium carotovora* DNA; lane 14, water blank.

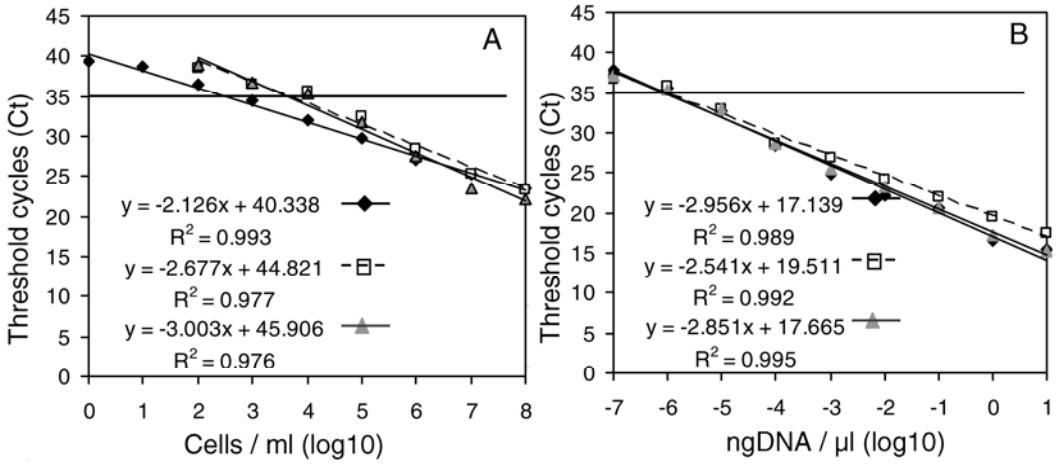

Fig. 2. Sensitivity of CelA (diamond), Cms50 (square), and Cms72a (triangle) polymerase chain reaction (PCR) primers used for detection of *Clavibacter michiganensis* subsp. *sepedonicus* bacterial cells **(A)** and DNA extracted from these bacterial cells **(B)** of plasmid positive strain OFF-1. Horizontal line represents 35 PCR cycles, the threshold used to make a positive or negative determination. Each data point is the mean of two trials at each concentration of DNA or bacterial cells for each PCR primer.

and presumably build up inoculum which allows subsequent spread (43). A further complication in the persistence of BRR is that cultivars continue to be developed that do not express disease symptoms in either foliage or tubers (14,27,34,35). Although the absence of disease expression is one means by which the pathogen can go undetected (34,35), seasonal variability in symptom development also occurs among susceptible cultivars in response to environmental conditions (27,33). Regardless of the reason, symptomless infections of *C. michiganensis* subsp. *sepedonicus* have proven insurmountable in the production of certified seed if visual inspections for the disease are relied upon. Additionally, this pathogen is capable of surviving protracted periods of time as dried slime on potato production surfaces, such as storage walls and equipment, making the pathogen extremely persistent on seed potato farms once they have become infested with the BRR bacterium (42). Noninfected certified seed that comes in contact with contaminated production surfaces can become infected, and the disease cycle begins again. This is particularly important in North America, where most seed is cut before planting, providing ideal infection courts for the ring rot bacterium. The net result is that BRR continues to be a major disease of potato and *C. michiganensis* subsp. *sepedonicus* remains a globally important quarantine pest, making reliable and highly sensitive methods to detect virulent *C. michiganensis* subsp. *sepedonicus* in certified seed a critical phase of pathogen and disease control.

Although a great deal of effort has been placed on the unambiguous identification and detection of the ring rot bacterium, there are significant differences in the methodology employed to determine sensitivity and specificity of these techniques. Studies on the identification and differentiation of *C. michiganensis* subsp. *sepedonicus* from other bacteria and bacterial plant pathogens have used in vitro grown bacteria almost exclusively (2,21,26,32, 49,51). Additionally, studies to improve detection have also only utilized in vitro grown bacteria (50). Studies designed to detect this pathogen in plant tissue tend to use artificially contaminated, or spiked, potato tissue (6,37) in addition to individual infected potato plants or eggplants (3,15,20,23,25,29–31,36,48). Unfortunately, few studies have been directed at the primary target for *C. michiganensis* subsp. *sepedonicus* detection technology, that being composite tuber samples (5,13,16). Postharvest testing of certified seed lots for the presence of *C. michiganensis* subsp. *sepedonicus* is an effective methodology for detecting asymptomatic

Table 4. Sensitivity of real-time polymerase chain reaction (PCR) using Cms50/72a and CelA primers for the detection of asymptomatic *Clavibacter michiganensis* subsp. *sepedonicus* infections in artificially produced composite tuber samples in 2006

		Number of asymptomatic tuber cores/total number of cores[a]					
		1/100		1/200		1/400	
Strain type	IFU[b]/gram tuber tissue	Cms50/72a[c]	CelA[c]	Cms50/72a[c]	CelA[c]	Cms50/72a[c]	CelA[c]
Mucoid	1.7×10^9	- (38.0/39.0)	- (37.8)	- (0/0)	- (0)	- (0/0)	- (0)
	2.0×10^7	- (0/0)	+ (31.0)	- (0/0)	- (0)	- (0/0)	+ (33.1)
	0	+ (28.7/30.0)	+ (26.1)	+ (31.8/32.9)	- (0)	- (36.3/37.8)	+ (34.6)
	0	- (0/0)	+ (29.7)	- (0/0)	+ (30.8)	- (0/0)	+ (33.9)
Nonmucoid	2.1×10^{10}	+ (34.2/34/6)	+ (28.4)	- (35.8/36.8)	+ (30.3)	- (38.3/38.6)	+ (33.9)
	1.0×10^7	- (38.6/38/9)	- (0)	- (0/0)	- (0)	- (0/0)	- (0)
	0	+ (31.0/32.3)	+ (33.9)	- (35.6/36.6)	- (35.8)	- (0/0)	- (39.4)
	0	- (36.3/37.3)	+ (29.2)	- (38.9/39.0)	+ (31.3)	- (0/0)	+ (34.6)

[a] Soak liquid from 100 presumed healthy cores tested negative for immunofluorescent-antibody staining (IFA), enzyme-linked immunosorbent assay (ELISA), as well as PCR with Cms50/72a and CelA. Therefore, healthy tuber soak fluid was included as a negative control for all assays. If a false positive was obtained in these negative samples, the assay was disregarded.
[b] Immunofluorescent units (IFU) per gram of tuber tissue. Tuber cores were soaked following standard protocols and supernatant was assayed.
[c] Positive or negative detection of *C. michiganensis* subsp. *sepedonicus* denoted as +/-, respectively. Mean crossing threshold values (Ct) of two assays are in parentheses. Samples with Ct values greater than 35 were considered negative.

Table 5. Sensitivity of real-time polymerase chain reaction (PCR) using Cms50/72a and CelA primers for the detection of asymptomatic *Clavibacter michiganensis* subsp. *sepedonicus* infections in artificially produced composite tuber samples in 2007

		Number of asymptomatic tuber cores/total number of cores[a]											
		1/100				1/200				1/400			
Strain type	IFU[b]/gram tuber tissue	IFA[c]	ELISA[d]	Cms50/72a[e]	CelA[e]	IFA[c]	ELISA[d]	Cms50/72a[e]	CelA[e]	IFA[c]	ELISA[d]	Cms50/72a[e]	CelA[e]
Mucoid	4.7×10^7	-	+ (0.303)	+ (28.0/28.6)	+ (23.8)	+	+ (0.359)	+ (31.2/32.4)	+ (25.6)	-	+ (0.345)	- (35.2/36.7)	+ (29.7)
	3.3×10^7	-	+ (0.448)	+ (29.5/30.7)	+ (27.1)	+	+ (0.392)	+ (33.5/34.7)	+ (29.1)	+	+ (0.445)	- (36.5/38.0)	+ (33.8)
	7.6×10^6	-	+ (0.211)	+ (33.0/34.1)	+ (32.5)	-	- (0.156)	- (35.5/36.7)	- (35.2)	-	- (0.149)	- (39.8/0)	- (38.7)
	3.1×10^6	-	+ (0.241)	+ (32.8/34.0)	+ (31.2)	-	- (0.160)	- (36.1/36.4)	+ (33.2)	-	- (0.161)	- (37.8/38.4)	- (36.7)
	1.5×10^5	+	+ (0.266)	+ (31.5/32.3)	+ (31.3)	-	- (0.189)	+ (33.3/34.1)	+ (32.1)	-	- (0.176)	- (36.2/36.6)	+ (34.3)
	0	-	+ (0.456)	+ (33.0/34.1)	+ (31.6)	+	+ (0.403)	- (36.0/37.1)	+ (32.7)	-	+ (0.385)	- (37.8/38.5)	+ (34.2)
Nonmucoid	8.1×10^8	-	- (0.121)	+ (30.9/32.6)	+ (24.0)	-	- (0.112)	+ (32.9/34.2)	+ (25.0)	-	- (0.112)	- (35.6/37.3)	+ (30.3)
	5.0×10^7	-	- (0.136)	+ (31.3/33.0)	+ (25.2)	+	- (0.111)	+ (33.6/34.8)	+ (27.3)	+	- (0.106)	- (36.8/37.4)	+ (31.0)
	2.2×10^7	-	- (0.110)	+ (30.0/31.8)	+ (26.8)	-	- (0.103)	+ (31.6/32.6)	+ (28.7)	-	- (.0104)	+ (33.7/34.0)	+ (32.6)
	3.0×10^6	-	- (0.115)	+ (32.5/33.4)	+ (29.9)	+	- (0.105)	- (36.3/36.0)	+ (32.2)	-	- (0.103)	- (38.3/38.8)	+ (34.0)
	0	-	- (0.108)	+ (30.1/31.8)	+ (30.6)	-	- (0.110)	+ (32.2/33.3)	+ (33.0)	-	- (0.106)	- (37.0/38.3)	+ (34.5)

[a] Soak liquid from 100 presumed healthy cores tested negative for immunofluorescent-antibody staining (IFA), enzyme-linked immunosorbent assay (ELISA), as well as PCR with Cms50/72a and CelA. Therefore, healthy tuber soak fluid was included as a negative control for all assays. If a false positive was obtained in these negative samples the assay was disregarded.
[b] Immunofluorescent units (IFU) per gram of tuber tissue. Tuber cores were soaked following standard protocols and supernatant was assayed. Tuber cores with zero IFU/g were tested via PCR with Cms50/72a and CelA to ensure infection by *C. michiganensis* subsp. *sepedonicus* had taken place.
[c] Samples with greater than 5 IFU/microscope field (MF) were considered positive.
[d] Mean absorbance values at 405 nm of triplicate wells is in parentheses. Samples with absorbance values greater than 0.2 at 405 nm were considered positive. Mean absorbance values for the positive and negative controls were 0.458 and 0.103, respectively.
[e] Positive or negative detection of *C. michiganensis* subsp. *sepedonicus* denoted as +/-, respectively. Mean crossing threshold values (Ct) of two assays are in parentheses. Samples with Ct values greater than 35 were considered negative.

infections of the ring rot bacterium, a procedure first suggested by De Boer (11,13). However, potato stems collected during the growing season can also be used to detect asymptomatic infections (23). Early postharvest testing studies were directed at determining the performance of serological methods such as ELISA and IFA (13,16), methods that are still recommended and employed today (1,12). While our research group has made a previous effort to develop PCR technology to improve detection sensitivity (5), ring rot infections in certified seed lots continue, indicating that more research is needed. Studies reported here clearly demonstrate that the real-time CelA PCR assay developed by our laboratory is robust compared to several other standard detection technologies for this pathogen. We believe this is the first time a *C. michiganensis* subsp. *sepedonicus* detection method has been developed from the onset with the primary objective of improving the detection of the ring rot bacterium in composite tuber samples.

The CelA primers reported here are specific to *C. michiganensis* subsp. *sepedonicus,* and we found no evidence that they will cross-react with any other potato pathogen or gram-positive plant pathogenic actinomycete. The data generated during these studies clearly demonstrate that the CelA primers are more sensitive than standard postharvest tests using monoclonal antibodies in ELISA and IFA (17,18). The CelA primers also proved to have greater sensitivity in the detection of symptomless *C. michiganensis* subsp. *sepedonicus* infection in composite seed lots compared to Cms50/72a with few exceptions. The Cms50/72 primers (37) are among the most widely used PCR primers to detect latent infections of the ring rot bacterium. The CelA primers detected *C. michiganensis* subsp. *sepedonicus* in artificially contaminated seed lots and in naturally occurring certified seed lots that were known to have been exposed to the pathogen with greater sensitivity than other detection methods. The CelA real-time PCR assay was as sensitive in detecting *C. michiganensis* subsp. *sepedonicus* in 1/400 composite tuber samples as other methods were in successfully detecting the ring rot bacterium in 1/100 or 1/200 composites. The CelA primers were frequently the only detection technology that successfully detected a symptomless infection after seed lots were monitored in the field during the growing season. Clearly, the CelA primers reported here have an advantage for use in postharvest testing compared to the current recommended methods (1,12). Furthermore, since 200 tuber composites are the global standard for seed lot indexing (1,12), real-time PCR assay using CelA should be an improvement over existing technology at composite sample numbers of 400, which could dramatically reduce the costs associated with such testing. There are a number of additional studies that need to be performed to enhance the utility of the CelA real-time PCR format to optimize the assay for the detection of asymptomatic infections. The CelA primers should either be multiplexed with Cms50/72a primers (37) to reduce the potential for false positives, or an internal reaction control can be integrated into the TaqMan PCR assay (55) in order to minimize false negative results.

During the course of these studies, we attempted to produce artificially infected composite tuber samples that were infected with differing levels of the target bacterium. We attempted to do this by quantifying the level of *C. michiganensis* subsp. *sepedonicus* in the BRR-infected tubers using IFA and selecting those infected tuber cores that had low to high levels of ring rot bacteria prior to blending them with healthy tuber cores. Although we were generally successful in achieving our goal of producing artificially infected composite tuber samples that differed in the level of *C. michiganensis* subsp. *sepedonicus*, there were notable exceptions (Table 4). In a few specific cases, high levels of *C. michiganensis* subsp. *sepedonicus* in infected tuber cores, as determined by IFA, were blended with healthy tuber cores, and the resulting PCR tests were negative for the pathogen. Conversely, in the same experiment, tuber cores that were IFA negative but PCR positive, which we interpreted as a low infection, tested positive with PCR. These incongruities are difficult to explain but may be due in part to the difficulties that can exist when bacterial cells adhere to tuber tissue and are obscured during microscopic examination of the IFA slides, which has been previously discussed (3,23). In other cases, the incongruity of these results may be due also to the presence of inhibitors to the PCR reaction that are known to exist in tuber tissue. Inhibitors to the PCR reaction have been previously reported and discussed in conjunction with *C. michiganensis* subsp. *sepedonicus* detection (5,52). Nonetheless, we do not believe these minor flaws in data set detract from the overall results we obtained, which clearly indicate real-time PCR is superior to serological techniques such as ELISA or IFA for the detection of symptomless infections of *C. michiganensis* subsp. *sepedonicus*. However, they do illustrate a potential pitfall of this type of testing and the care that must be taken to reduce or eliminate potential inhibitors to the PCR reaction.

It is clear from the data in studies reported here that ELISA is ineffective in detecting symptomless infections of *C. michiganensis* subsp. *sepedonicus* when indexing seed lots. In controlled experiments, ELISA was effective in detecting symptomless infections only when composite samples of 1/100 were used and only with mucoid strains of the pathogen. When certified seed lots known to be exposed to *C. michiganensis* subsp. *sepedonicus* were indexed in 2006, ELISA only detected the pathogen in four of the seed lots tested, whereas IFA, Cms50/72, and CelA detected *C. michiganensis* subsp. *sepedonicus* in 7, 10, and 10 seedlots, re-

Table 6. Indexing of certified seed potatoes exposed to bacterial ring rot (BRR) in 2006[a]

		Results of testing (no. positive/no. tested)[b]				
Seed farm	**Seed lot**	**ELISA (abs)[c]**	**IFA**	**Cms50/72a**	**CelA**	**BRR (%)[d]**
A	1	0/19 (0.008-0.029)	0/19	0/19	0/19	0
A	2	0/20 (0.002-0.012)	0/20	0/20	0/20	0
A	3	2/21 (0.010-0.291)	2/21	2/21	2/21	4.5-6.0
A	4	0/20 (0.007-0.019)	0/20	0/20	0/20	0
B	1	0/19 (0.001-0.010)	0/19	0/19	0/19	0.1
B	2	0/20 (0.006-0.054)	1/20	5/20	13/19	8.5-10.0
B	3	0/16 (0.001-0.008)	0/16	0/16	0/16	0
B	4	0/19 (0.002-0.011)	0/19	1/19	1/19	0
B	5	0/19 (0.008-0.014)	0/19	0/19	0/19	0
B	6	0/22 (0.002-0.035)	0/22	0/22	0/22	0
C	1	0/22 (0.001-0.028)	0/22	1/22	5/22	6.5-8.0
C	2	1/10 (0.001-0.331)	1/10	4/10	7/10	15-29
C	3	1/22 (0.008-0.330)	1/22	3/22	3/22	5-6
C	4	1/16 (0.012-0.331)	1/16	1/16	1/16	6-8
C	5	0/20 (NA)	2/20	6/20	13/20	4-6
C	6	0/20 (NA)	0/20	8/20	14/20	17-20
C	7	0/20 (NA)	1/20	1/20	5/20	6-9

[a] Healthy tuber soak fluid was included as a negative control for all assays. If a false positive was obtained is these negative samples the assay was disregarded.

[b] Numbers of tubers submitted for BRR testing varied from 3,200 to 4,400 per seed lot. Tests for the presence of *Clavibacter michiganensis* subsp. *sepedonicus* were performed on stem end cores taken from 200 tubers per composited sample.

[c] Numbers of enzyme-linked immunosorbent assay (ELISA) positives per number of composites tested. Range of absorbance values at 405 nm is in parentheses. Samples with absorbance values greater than 0.2 at 405 nm were considered positive. Mean absorbance values for positive and negative controls were 0.576 and 0.043, respectively.

[d] Estimated percentage of BRR expressed in the field the year following laboratory testing. A minimum of 1,000 plants were rated for symptoms.

spectively. The number of composite tuber samples that tested positive with CelA versus the other diagnostic tests was also substantially higher, which is further evidence that these primers are very robust when used in a real-time PCR format. Although the potato growers involved in this portion of the study were cautioned against using these seed lots in the production of their crop, they chose to do so because of potato seed shortages. The high incidence of BRR observed in the fields grown with this seed was undoubtedly due to the seed cutting and handling procedures employed by these growers. However, the fact remains that CelA real-time PCR was the only method that was able to detect potentially high infection rates in these seedlots compared to the other diagnostic methodologies used during this portion of our studies.

The cellulase A virulence gene sequence is on the plasmid of the *C. michiganensis* subsp. *sepedonicus* strain that was genome sequenced (7). However, not all strains have a plasmid (40,41). Early studies demonstrated that pCS1 and a repeated sequence from this plasmid were highly conserved with approximately 1.26 to 1.5 copies in each *C. michiganensis* subsp. *sepedonicus* strain depending on whether or not it is integrated into the chromosome or episomal, respectively. This repeated sequence was the target of early attempts to improve detection with either DNA hybridization or PCR (20,54), but those early diagnostic methods proved to be no more sensitive than serological assays employed at the time. One of the initial concerns in the development of the CelA primers for *C. michiganensis* subsp. *sepedonicus* detection was the fact that the cellulase pathogenicity genes are present on the plasmid pCS1 (7), and this native plasmid may either be autonomous or in integrated form (39,41). Although rare, there are two strains of *C. michiganensis* subsp. *sepedonicus* in which pCS1 is not present either episomally or integrated, P45 and CS14 (41). Strain P45 has received considerable attention as a result (28,45). It has been suggested that the P45 strain lost its native plasmid after nearly 40 years of subculturing (53). Strain CS14, originally recovered from infected potato tubers from Montana, has been in our collection for nearly 30 years and was obtained from two independent sources. Our initial concern was that strains such as P45 and CS14 would not be detected by the CelA primers described here, a phenomenon noted during the development of other pCS1-based DNA probes (40) and PCR primers (53). Results of the studies reported here confirm our initial concern that the CelA PCR primers do not detect strains P45 and CS14, which have been reported as avirulent (41). However, it should be noted that our P45 culture, originally obtained many years ago from S. H. De Boer, has been demonstrated to contain pCS1 (50). If the results of that research were correct, we would expect the CelA primer to detect strain P45, but they did not. There is no evidence we are aware of that suggests that strains such as P45 and CS14 exist in nature, and we are inclined to agree with previous investigators (53) that these strains are anomalies created by many years of subculturing. Indeed, the screening of numerous strains from our culture collection and 4 years of field testing natural infections have led us to believe that the CelA primers described here are specific to virulent *C. michiganensis* subsp. *sepedonicus* with improved sensitivity. The ability of CelA primers to detect *C. michiganensis* subsp. *sepedonicus* strains that are plasmid negative indicates that the CelA gene is integrated into the chromosomes of naturally occurring plasmid negative strains. Mogen et al. (41) found pCS1 either free or integrated in the form of a repeated sequence in most strains of *C. michiganensis* subsp. *sepedonicus,* causing the authors to speculate that the repeated sequence must serve some important function for the pathogen that was at the time unknown. It is likely that function is pathogenicity, but that can only be confirmed with further genome sequencing of *C. michiganensis* subsp. *sepedonicus* strains known to be negative for pCS1.

This is the first study we are aware of that compares the sensitivity of two serological and two PCR methods in the detection of *C. michiganensis* subsp. *sepedonicus* in composite tuber samples. We were careful to use large sample sizes, usually 4,000 tubers per seed lot, when testing certified seed lots that had been exposed naturally to *C. michiganensis* subsp. *sepedonicus*. These samples sizes are consistent with those recommended in a statistical analysis performed by Clayton and Slack (10) specifically with BRR in mind. Despite using large sample sizes, ELISA and IFA were ineffective in detecting latent *C. michiganensis* subsp. *sepedonicus* infections in several seed lots, even though the grow outs of these seed sources demonstrated relatively high infection rates. Since ELISA and IFA demonstrated low frequencies of detection in composite samples, relative to CelA or Cms50/72 PCR, we recommend that real-time PCR technology be used as the primary screening tool when indexing certified seed lots for the presence of *C. michiganensis* subsp. *sepedonicus*.

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