An Internal Reaction Control for Routine Detection of *Clavibacter michiganensis* subsp. *sepedonicus* Using a Real-Time TaqMan PCR-Based Assay

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ABSTRACT

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An internal reaction control was integrated into a TaqMan polymerase chain reaction (PCR) assay for the detection of *Clavibacter michiganensis* subsp. *sepedonicus*, the causal organism of bacterial ring rot of potato. The reaction control, cloned into plasmid pCmsC4, consisted of a sequence unrelated to *C. michiganensis* subsp. *sepedonicus* flanked by the primer sequences used in the TaqMan PCR, thus eliminating the need for multiplexing. Inclusion of the reaction control plasmid in the TaqMan assay had no effect on either the limit of detection or the specificity of the method. Addition of SYBR Green permitted melt analysis of PCR products. The 242-bp reaction control amplicon, with a melt temperature of approximately 94.5°C, could easily be distinguished from the 152-bp primary diagnostic target amplicon, which had a melt temperature of about 85.5°C. Electrophoretic analysis showed that appearance of either melt peak correlated well with the presence of the appropriate amplicon. Two different substances, guanidine-HCl and humic acid, inhibited the amplification of the reaction control at concentrations lower than those that inhibited the primary diagnostic target, demonstrating the reaction control's effectiveness in detecting inhibition or reaction failure. Using the reaction control plasmid, a quantitative threshold for inhibitor detection was established. This permitted the validation of negative results, and thus facilitated the use of TaqMan real-time PCR in the routine testing of diagnostic samples for *C. michiganensis* subsp. *sepedonicus.*

Clavibacter michiganensis subsp. *sepedonicus* causes bacterial ring rot of potato (*Solanum tuberosum* L.), a disease that warrants serious concern because of its potential to cause significant economic losses. *C. michiganensis* subsp. *sepedonicus* is readily spread through seed potato tubers, particularly by latent infections in which a low incidence of inoculum persists asymptomatically in potato for several generations (11,18). Because sound cultural practices including compliance with strict phytosanitary measures is the only means of controlling the spread of this pathogen, seed certification programs have been implemented in most jurisdictions where seed potatoes are produced.

To support regulatory enforcement, valid and robust laboratory methods are required for the detection of subsymptomatic levels of *C. michiganensis* subsp. *sepedonicus*. Enzyme-linked immunosorbent assays (ELISA) utilizing a monoclonal antibody (9) are widely used, as they are well suited to large-scale, high-throughput screening, and the required antibodies and reagents are commercially available. Immunofluorescence microscopy (IMF) is also used, sometimes as a confirmatory test for ELISA-positive samples, because it enables the direct visualization of *C. michiganensis* subsp. *sepedonicus* cells (8). IMF is more labor-intensive than ELISA, and interpretation can be subjective.

Nucleic acid–based tests offer an alternative to serological-based detection of plant pathogens, and the comparative advantages and disadvantages of each have been recently reviewed (29). A number of polymerase chain reaction (PCR)-based methods for detection of *C. michiganensis* subsp. *sepedonicus* have been published (10,14,15,17,19,25). Such DNA amplification-based methods offer much lower limits of detection, but despite the level of specificity and sensitivity that can be achieved with PCR, there are two major factors that have adversely affected its value as a diagnostic tool in a regulatory context. The first is the occurrence of false negative results due to reagent or thermocycler failure or to the presence of co-extracting substances in the sample that inhibit the polymerase reaction (28). Potato starch, acidic polysaccharides, and phenolic compounds may be present in tuber or stem extracts, and are known to inhibit polymerase reactions (2). Fungal polyphosphates and humic acid from soil, both potent inhibitors of PCR (2), may also be associated with potato tissue and be co-extracted with DNA.

An internal reaction control can be incorporated into a PCR test to validate negative test results (21). Of several conventional PCR methods for the specific detection of *C. michiganensis* subsp. *sepedonicus,* the first to include an internal reaction control was a competitive PCR in which an unrelated sequence was added to the reaction mix to be co-amplified with target DNA by the target-specific primers (12). In a later method, an endogenous gene, 18S rRNA, served as an internal reaction control, and was co-amplified using a separate set of primers in a multiplex reaction (20). This approach was also applied for the detection of *Ralstonia solanacearum*, in which a plastid gene, cytochrome oxidase, was used as an internal reaction control (30).

The second factor limiting the utility of PCR for regulatory work is the significant potential for amplicon carry-over into samples or reagents, which can cause the generation of false positive results (28). Conventional PCR methods require postamplification manipulation, including electrophoresis and hybridization analyses, but such manipulation can result in the dispersal of amplicons throughout the laboratory, contributing to the risk of false positives. The implementation of real-time fluorescence PCR methods, in which the amplicons are detected using double-stranded DNA binding dyes or fluorescent-labeled, sequence-specific probes, eliminates the need to open the reaction tube.

Real-time PCR, with TaqMan probes (16) that utilize the 5′ exonuclease activity of *Taq* polymerase to separate quencher and fluorophore moieties on labeled probes, permits measurement of amplified product as the reaction proceeds. By adding a DNA-binding dye such as SYBR Green, postreaction melt analysis of amplicons can also be conducted to confirm amplicon identity directly in the reaction tube (22). At least three real-time amplification methods for the detection of *C. michiganensis* subsp. *sepedonicus* have been published. A nucleic acid sequence based amplification (NASBA) method, designed for epidemiological studies, en-

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*The *e*-Xtra logo stands for "electronic extra" and indicates that Figure 8 appears in color in the online edition.

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abled the detection of viable *C. michiganensis* subsp. *sepedonicus* cells (27). A second method targeting the intergenic ribosomal DNA region and utilizing conserved primers and variable TaqMan probes was developed for differentiating subspecies of *C. michiganensis* (1). A third method was formulated as a real-time BIO-PCR assay in which target bacteria are multiplied in a semi-selective medium prior to DNA extraction (24).

The objective of this study was to incorporate an internal reaction control in a real-time TaqMan PCR method based on published primer and probe sequences (24), and to specify a threshold for validating negative test results. A postreaction melt analysis step was incorporated as part of routine testing to confirm amplicon identities within a closed-tube system. The results of this assay were evaluated in comparison to results obtained by serological detection methods conducted within a quality assurance testing program.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *C. michiganensis* subsp. *sepedonicus* strain R14 was used as a positive control and in the spike-recovery experiments. The bacterium was maintained in Nutrient Broth (BD Difco, Sparks, MD) with 50% glycerol at –70°C, and cultured or dilution-plated for enumeration on yeast extract-glucose-mineral salts medium (YGM) (5). Plates were incubated at room temperature (ca. 21°C) for 2 to 4 days before use. For DNA preparations where the cells were not enumerated, single colonies were resuspended in sterile water to form a visibly turbid suspension.

Escherichia coli strain DH5α-T1 was purchased from Invitrogen (Carlsbad, CA). Transformant clones derived from this strain were grown in Luria-Bertani (LB) broth (BD Difco) or LB agar supplemented with 50 μg/ml ampicillin (Roche Applied Sciences, Laval, Canada) at 37°C for 16 to 24 h.

Custom oligonucleotides. With the exception of the TaqMan probe 50-53T, which was supplied by Integrated DNA Technologies (Coralville, IA), all oligonucleotides were prepared-to-order by Eurogentec (San Diego, CA). Sequences of all the oligonucleotides along with their source references are listed in Table 1. All original oligonucleotide sequences developed for use in this study were designed using Clone Manager Suite v. 6.00 (Science and Educational Software, Cary, NC).

Potato sample material and preparation of homogenates. Potato tuber samples from discrete production lots were submitted to the laboratory from farm units in various regions across Canada. Cores of vascular tissue (0.4 to 1.0 g) were taken from the stolon end of each tuber, washed, weighed, and homogenized in distilled water at a rate of 1 ml/g tissue using a blender (Waring, Torrington, CT). Typically, samples were composite, each comprised of 200 cores homogenized together. The homogenates were filtered through a double layer of cheesecloth and stored frozen in 50-ml, screw-capped polypropylene tubes until further use.

Total DNA extraction. Bacterial cells in pure culture or in potato homogenates were lysed by mixing 500 μl homogenate or culture suspension with 200 μl of a stock extraction buffer (175 mM Tris-HCl, pH 8.0, 8.75 mM ethylenediamine tetracetic acid, 3.5% sodium dodecyl sulfate, and 35 μg/ml proteinase K) and incubating 3 h to overnight at 55°C. DNA was extracted using the components from the Magnesil KF DNA extraction kit (Promega, Madison, WI). Initially, 500 μl of digest was mixed with an equal volume of lysis buffer and 100 μl of magnetic beads. The remaining kit components were used to wash and elute the DNA with a Kingfisher magnetic particle processor (Thermo Scientific, Waltham, MA) according to the kit instructions. The extracts were transferred into sterile, nuclease-free microfuge tubes and stored at –20°C or colder until analyzed.

A few samples required postextraction cleanup to remove residual inhibitors. These extracts were diluted into 600 μl of Ultraclean Plant DNA buffer P3 (Mo-Bio, Carlsbad, CA) and processed in spin columns (Mo-Bio) according to the manufacture's instructions to obtain a purified DNA fraction.

Construction of the reaction control plasmid pCmsC4. An overview of the strategy used to construct pCmsC4 is shown in Figure 1. The reaction control sequence was prepared from three fragments. The left fragment (left arm) and right fragment (right arm) contained the *C. michiganensis* subsp. *sepedonicus*–specific primer sequences 50-2F and 133R, and were generated in PCR from 100-mer synthetic single-stranded oligos using primers ivr-f1 and ivr-r1. The middle fragment (495 bp) was generated in PCR from the corn (*Zea mays* L.) invertase gene, also using primers ivr-f1 and ivr-r1. The extraction method used to prepare the genomic DNA has been previously described (26).

The 100-bp left and right arm fragments were digested with 0.4 units/μl of *Mlu*I and *Hae*II, respectively (New England Biolabs, Ipswich, MA). The resulting 73-bp fragments were both gel-purified using a Qiaquick gel extraction kit (Qiagen, Mississauga, Canada). The 495-bp invertase fragment was double digested with *Mlu*I and *Hae*II, and the resulting 185-bp fragment was ligated to the two 73-bp fragments using T4 ligase (Roche Applied Science).

The ligation products were analyzed on a 2% agarose gel, and a 400- to 450-bp product was identified and purified. The desired ca. 240-bp fragment (Fig. 1) was amplified from 1 μl of this preparation in PCR using primers 50-2F and 133R, and cloned into pCRII-TOPO (Invitrogen) according to the supplier's instructions. Competent cells of *E. coli* strain DH5α-T1 (Invitrogen) were transformed with 4 μl of the cloning reaction, and transformed colonies were selected and cultured for further analysis and characterization.

Plasmid purification and characterization. Plasmids were prepared from overnight cultures of single colonies grown from transformed cells using Wizard miniprep reagents and Wizard Resin (Promega), and quantified using Picogreen (Invitrogen) fluorescence. Preparations were

Table 1. Sequences of oligonucleotides (5′ to 3′) used in this study

Oligo	Sequence	Function	Reference
ivr-f1	ccggatcgtcatgctctaca	Forward PCR primer for corn gene *ivr1*, insert left arm, and reverse primer for insert right arm (Fig. 1)	This study
ivr-r1	gtgccgatcgcgtagtagtc	Reverse PCR primer for corn gene *ivr1*, insert left arm, and forward primer for insert right arm (Fig. 1)	This study
Insert left arm	ccggatcgtcatgctctacacggaagcttaggcggagtcgtccggagcgcgata gaagaggaactctttgtcacgcgtatgactactacgcgatcggcac	Left (5′) portion of artificial reaction control insert (Fig. 1)	This study
Insert right arm	gtgccgatcgcgtagtagtcgaagaggacgactctagagtggagcccgaggca gagcatcgctcagtacccaggcgccattgtagagcatgacgatccgg	Right (3′) portion of artificial reaction control insert (Fig. 1)	This study
50-2F	cggagcgcgatagaagagga	Forward PCR primer for Cms[a] detection	24
133R	ggcagagcatcgctcagtacc	Reverse PCR primer for Cms detection	24
50-53T	Cy5–aaggaagtcgtcggatgaagatgcg–Iowa Black RQ	Dual-labeled TaqMan probe for Cms detection	24
M13R	caggaaacagctatgac	Forward primer for sequencing pCmsC4 insert	Unknown
M13F(-20)	gtaaaacgacggccag	Reverse primer for sequencing pCmsC4 insert	Unknown

[a] Cms: primary diagnostic target *Clavibacter michiganensis* subsp. *sepedonicus*.

evaluated for stability, expected insert size, and ability to generate the desired 240-bp fragment in the TaqMan assay. Plasmid pCmsC4 was selected as the most suitable, and the insert fragment was sequenced by the Core Molecular Biology Facility of York University (Toronto, Canada) using primers M13F(-20) and M13R (Table 1).

For use as a reaction control in the TaqMan assay for *C. michiganensis* subsp. *sepedonicus,* pCmsC4 was linearized with 0.4 units/µl *Bgl*II (New England Biolabs). The linearized plasmid was quantified and stored as a 100× stock solution in 0.1× TE, pH 8.0. The number of copies per microliter was estimated from the mass concentration (ng/µl) according to the following formula:

$$\text{Copies/µl} = (\text{ng/µl}) \times (\text{nmol/650 ng bp}) \times 1/4{,}215 \text{ bp} \times 6.02 \times 10^{14} \text{ copies/nmol}$$

or

$$\text{Copies/µl} = \text{ng/µl} \times 2.2 \times 10^{8}$$

TaqMan assay for *C. michiganensis* subsp. *sepedonicus.* A previously published TaqMan assay (24) was optimized for the SmartCycler II (Cepheid, Sunnyvale, CA) fluorescence thermocycler. The reaction mix consisted of 1× SYBR Green JumpStart Taq ReadyMix (Sigma, St. Louis, MO), and 0.2% BLOTTO to help counteract inhibitor effects (7). The forward and reverse primers, 50-2F and 133R, respectively, were used at a concentration of 0.3 µM each. The TaqMan probe 50-53T was labeled at the 5′ end with Cy5 and at the 3′ end with Iowa Black RQ, and was used at a concentration of 0.2 µM. Linearized reaction control plasmid pCmsC4 was also included in the reaction mix at a final concentration of 4 copies/µl, or 100 copies per reaction. Two microliters of either sample template DNA, control DNA, or water (no template control) was also added. The reaction was carried out as follows: 1 cycle of 2 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 60 s at 64°C. Fluorescence was captured in the FAM and Cy5 channels during this step. The FAM channel was configured to excite and capture SYBR Green fluorescence, while the Cy5 channel was used to excite and capture Cy5 fluorescence. Following a final extension at 72°C for 3 min, melt curves were generated using the default settings.

The cycle threshold (Ct), the cycle at which the fluorescence rose significantly above background, was arbitrarily set at fluorescence values of 15 and 4 for the FAM and Cy5 channels, respectively, to facilitate between-run comparisons. Reaction efficiencies were determined by measuring the slope of the regression line generated by Ct value versus the log of the target concentration. Efficiency (E) was calculated as:

$$E = 1 - 10^{1/\text{-slope}} \qquad (23)$$

Selected samples were also analyzed by gel electrophoresis in 2% agarose to evaluate PCR products. The agarose gels contained 0.5 µg/ml ethidium bromide (Invitrogen), and were prepared and run in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA). Digital images of the gels were viewed and captured using the GeneGenius BioImaging system (Syngene, Cambridge, UK).

Effect of inhibitors on reaction control performance. Either humic acid (technical grade, Sigma) or guanidine hydrochloride (Sigma) was added at varying concentrations to the TaqMan assay. Templates for the assay were derived from samples that contained the equivalent of 0, 5×10^3, or 5×10^4 cells/ml of homogenate. The effect of the inhibitors on the reaction control plasmid pCmsC4 amplification and on *C. michiganensis* subsp. *sepedonicus* detection was determined by measuring the change of Ct (ΔCt) in the FAM and Cy5 channel, respectively, with and without the inhibitor.

Serological detection. *C. michiganensis* subsp. *sepedonicus* was detected using a triple antibody sandwich (TAS)-ELISA that utilized monoclonal antibody 1H3 as the primary detection antibody. Samples generating positive results in ELISA were also tested with IMF utilizing monoclonal antibody 9A1. Both methods were carried out according to standard protocols described in detail elsewhere (6,8,9). The threshold for IMF was 150 fluorescing cells/30 microscope fields, while the thresholds for the ELISA tests were calculated from positive and negative control values as previously described (4).

Statistical analyses. The D'Agostino and Pearson omnibus normality test was used to determine Gaussian goodness-of-fit for the frequency distributions. Grubbs' test was used to detect outliers, and was performed using an online calculator developed by GraphPad Software (San Diego, CA). All regression and statistical analyses, except Grubbs' test, were performed using GraphPad Prism version 4.03 (GraphPad Software).

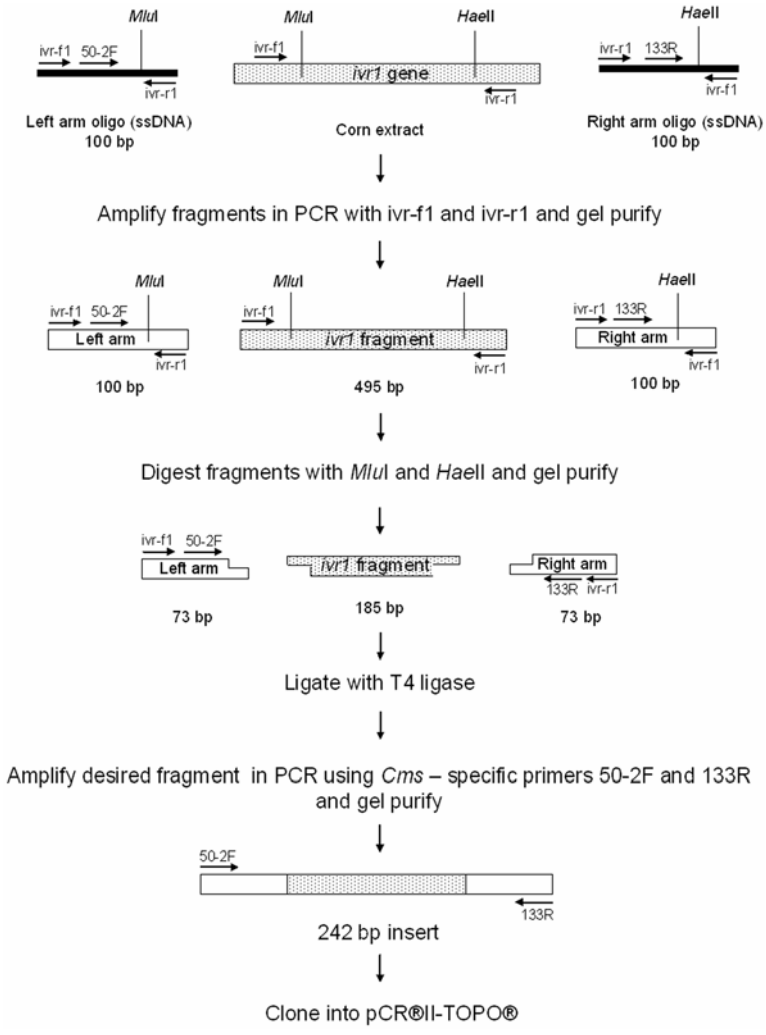

Fig. 1. Strategy used to construct the artificial reaction control plasmid pCmsC4.

RESULTS AND DISCUSSION

Analysis of pCmsC4. The reaction control plasmid, pCmsC4, contained a 242-bp insert sequence in forward orientation, and was flanked at the 5′ and 3′ ends by *C. michiganensis* subsp. *sepedonicus*–specific primer sequences 50-2F and 133R, respec-

tively (Fig. 2). The *Hae*II restriction site was retained in the insert, whereas the *Mlu*I site was lost in the ligation. The insert had a GC content of 73%.

The pCmsC4 insert sequence was amplified in the TaqMan assay using the primer pair 50-2F and 133R. The 242-bp reaction control amplicon was easily distinguishable from the 152-bp *C. michiganensis* subsp. *sepedonicus*–specific product in agarose electrophoresis (Fig. 3A). Addition of SYBR Green to the reaction mix allowed the identification of both products based on their melting profiles. The *C. michiganensis* subsp. *sepedonicus*–specific target amplicon, generated using a DNA extract from a pure culture of *C. michiganensis* subsp. *sepedonicus*, had a melting temperature of about 85°C; while the pCmsC4 reaction control amplicon, generated using pCmsC4 as template, had a melting temperature of approximately 94°C (Fig. 3B).

The SYBR Green (FAM Channel) Cts generated with the pCmsC4 template were inversely proportional to the log concentration of the plasmid DNA in the reaction (Fig. 4A), and the regression line had a correlation coefficient >0.99. This was the expected result, as it reflected the exponential nature of the amplification reaction. The slope of the regression line (Fig. 4A) was –3.801 ± 0.054, and on this basis the amplification efficiency of the pCmsC4 insert sequence in the TaqMan assay was calculated to be 83.2%.

Effect of reaction control on the TaqMan assay. DNA extracted from homogenates of healthy tuber tissue, to which had been added different concentrations of a pure culture of *C. michiganensis* subsp. *sepedonicus* from a 10-fold dilution series (10^4 to 10^8 CFU/ml), was used as the sample template in the TaqMan assay with and without the addition of 100 copies of linearized pCmsC4 per reaction. The Cy5 Cts generated from the TaqMan probe, with or without the reaction control, were very similar at each concentration of *C. michiganensis* subsp. *sepedonicus* (Fig. 4B). The regression lines generated with 0 and 100 copies of pCmsC4 per reaction were almost identical, with slopes of –3.55 ± 0.12 (r^2 = 0.99) and –3.48 ± 0.14 (r^2 = 0.99), respectively (Fig. 4B). Analysis of covariance indicated that there was no significant difference between the two slopes (F(1,16 df) = 0.147; P = 0.71). This demonstrated that the addition of 100 copies per reaction of pCmsC4 to the TaqMan assay did not affect the reaction efficiency, and was a strong indication that the addition of pCmC4 did not affect the sensitivity of the TaqMan assay. The mean efficiency of *C. michiganensis* subsp. *sepedonicus* target amplification calculated from the pooled slopes (–3.515) was 92.5%.

The limit of reliable detection of this TaqMan method was previously estimated at approximately 2 – 4 × 10^3 cells/ml homogenate (unpublished data). To test whether addition of the internal reaction control had an effect on the detection limit, a pure culture of *C. michiganensis* subsp. *sepedonicus* was spiked into healthy potato tuber homogenates at 10^3 CFU/ml, a concentration just below the reliable limit of detection. The spiked homogenates were extracted and analyzed for *C. michiganensis* subsp. *sepedonicus* using the TaqMan assay with either 0 or 100 copies of pCmsC4 in each reaction. The range of Cts obtained in the presence of 100 copies of pCmsC4 was 35.77 to 37.21, which was slightly higher than the range of Cts (34.09 to 36.13) obtained with no pCmsC4 present (Table 2). In the absence of the pCmsC4 reaction control, however, *C. michiganensis* subsp. *sepedonicus* was detected in the extracts in 3 out of 7 analyses; while in the presence of 100 copies of pCmsC4, *C. michiganensis* subsp. *sepedonicus* was detected in the extracts in 4 of 7 analyses (Table 2).

The stochastic nature of these results is often observed in PCR when target concentration in the extract is very low, and reflects the sampling error inherent in capturing the target template from the extract in a small subsampled volume for PCR analysis. The fact that there was little difference in the overall detection rate, with or without pCmsC4, supported the conclusion drawn from the data presented in Figure 4B; i.e., that the reaction control did not effectively compete with the primary diagnostic target in PCR, and ultimately had no adverse effect on the detection of *C. michiganensis* subsp. *sepedonicus*.

To evaluate the effect of pCmsC4 on the specificity of the TaqMan assay, 28 known positive and 50 known negative samples were analyzed for *C. michiganensis* subsp. *sepedonicus* in the presence of 100 copies of pCmsC4/reaction. All 28 positive samples generated positive results, while the 50 negative samples all returned negative results (Table 3). The reaction control plasmid pCmsC4, therefore, had no effect on the specificity of the TaqMan assay.

Result confirmation: defining amplicon melting temperature tolerances. Figure 3B demonstrated that the amplicons generated from the primary *C. michiganensis* subsp. *sepedonicus* target and the reaction control pCmsC4 could be easily distinguished by their melting temperature profiles. In order to use melting temperatures for confirming amplicon identity in a diagnostic application, temperature tolerances, or the range of acceptable melt peak temperatures, had to be established. In the case of both the positive and negative results, the accepted temperature range to confirm the presence of the correct ampli-

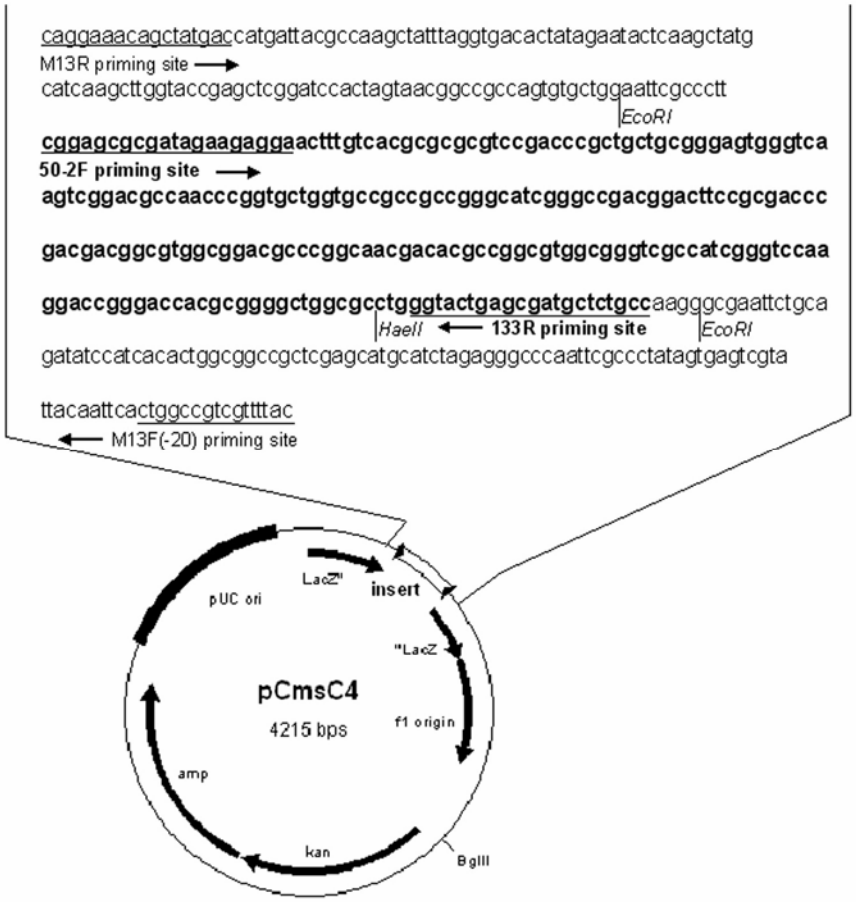

Fig. 2. Map and partial sequence of reaction control plasmid pCmsC4. Regular face type indicates the vector sequence while bold face type indicates the insert sequence. Primer binding sites are underlined.

con was defined as the range of temperatures that would include 99% of all true positive or negative results, i.e., the mean plus or minus 2.576 times the standard deviation. This is based on the fact that 1.00% of a standard two-tailed Gaussian distribution has *Z* values greater than 2.576 or less than –2.576, with $Z = 1$ standard deviation around the mean of the distribution.

Melt peaks generated by the 27 positive samples fit a normal distribution ($P = 0.94$; Fig. 5A), with a mean of 85.50°C and a standard deviation of 0.15°C. One outlier was detected (Fig. 5A). The accepted temperature range to confirm the presence of the *C. michiganensis* subsp. *sepedonicus*–specific 152-bp amplicon was therefore 85.11 to 85.89°C.

Of the 50 negative samples, 47 generated detectable melt peaks for the pCmsC4 reaction control amplicon (Table 3), and these, along with data from an additional 13 negative samples, were used to generated a frequency distribution histogram for the reaction control amplicon melt peak temperature (Fig. 5B). This data also fit a normal distribution ($P = 0.32$) with a mean melt peak temperature of 94.53°C and a standard deviation of 0.13°C. The accepted temperature range for the identification of the pCmsC4 reaction control amplicon was therefore 94.20 to 94.86°C.

Detecting reaction inhibitors. The main purpose of including a reaction control in a PCR analysis is to detect any potential inhibitors that may invalidate negative results. Experiments were performed in order to determine whether pCmsC4 was effective in indicating the presence of inhibitors or reaction failure in the TaqMan assay. Addition of either humic acid or guanidine hydrochloride increased the Cts generated by both pCmsC4 and the *C. michiganensis* subsp. *sepedonicus* target in a concentration-dependent manner (Fig. 6), indicating that these substances had an inhibitory effect on the amplification of both targets. The plateaus of the curves at the higher concentrations indicated that the Cts could not increase further, as the reaction was completely inhibited (Fig. 6).

For humic acid, 0.25 µg/ml was sufficient to increase the Ct of the pCmsC4 RC by 8 cycles, while the Ct generated by the *C. michiganensis* subsp. *sepedonicus* target at either 5×10^3 or 5×10^4 cells/ml increased by a little more than 1 cycle (Fig. 6A). The addition of 0.3 µg/ml humic acid increased the pCmsC4 RC Ct by more than 10 cycles, completely inhibiting the reaction, while increasing the Cts generated by the *C. michiganensis* subsp. *sepedonicus* target by only about 2 cycles. The addition of more than 0.4 µg/ml humic acid was required to completely inhibit the amplification of the *C. michiganensis* subsp. *sepedonicus* target and generate a false negative result.

Guanidine hydrochloride at 11 mM increased the pCmsC4 Ct by about 7 cycles

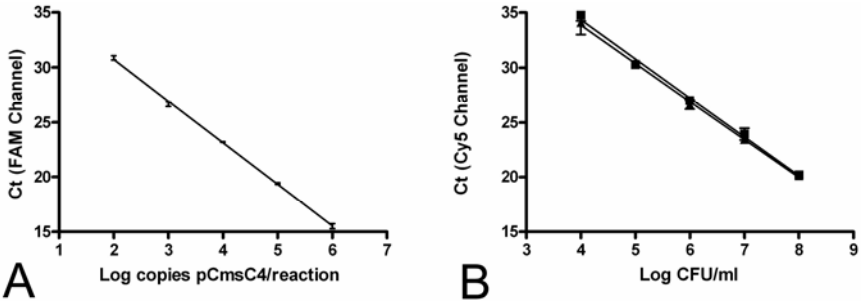

Fig. 4. Cycle thresholds (Cts) generated in the **(A)** FAM channel as a function of the concentration of pCmsC4 and in the **(B)** Cy5 channel as a function of the concentration of the primary assay target, *Clavibacter michiganensis* subsp. *sepedonicus*, with 0 (■) and 100 (▲) copies of pCmsC4 added per reaction. Error bars represent the standard deviation around the mean of duplicate observations **(B)** in two runs **(A)**.

Table 2. Detection of very low levels (ca. 10^3 CFU/ml[a]) of *Clavibacter michiganensis* subsp. *sepedonicus* in various extracts, with and without the positive reaction control pCmsC4 spike

	Ct in Cy5 Channel	
Extract	**0 copies pCmsC4/reaction**	**100 copies pCmsC4/reaction**
1	>40 (not detected)	35.32
2	36.02	37.21
3	>40 (not detected)	>40 (not detected)
4	>40 (not detected)	>40 (not detected)
5	>40 (not detected)	36.26
6a[b]	36.13	35.11
6b[b]	34.09	>40 (not detected)
Positive control	17.09	17.33
No template control	>40 (not detected)	>40 (not detected)

[a] Below the reliable limit of detection.
[b] Duplicate observations with the same extract.

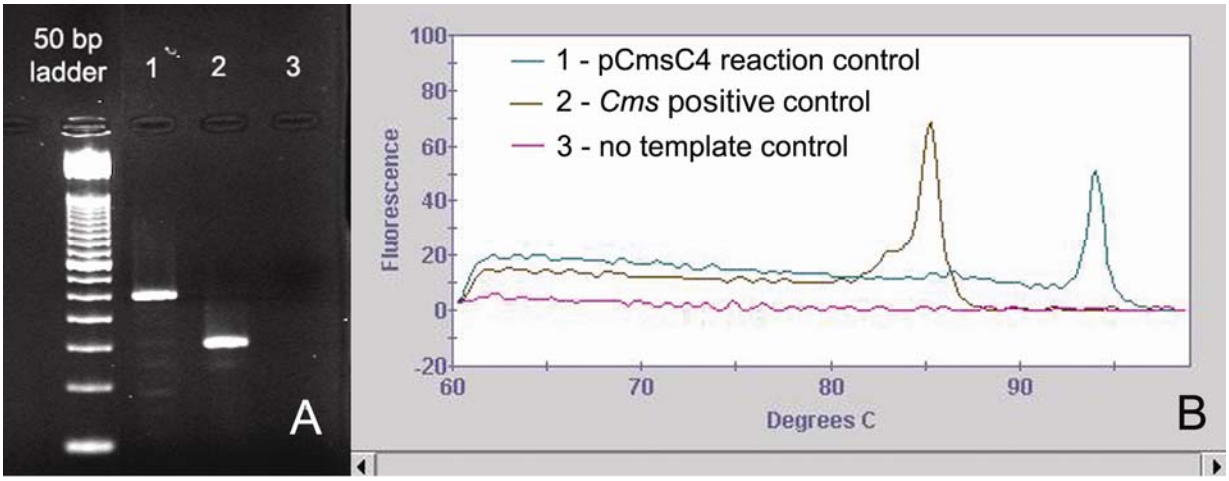

Fig. 3. Characterization of amplicons produced in real-time polymerase chain reaction (PCR) with the following templates: (1) pCmsC4 reaction control; (2) DNA extract from a pure culture of *Clavibacter michiganensis* subsp. *sepedonicus* (Cms); (3) nuclease-free water (no template control) in **A,** 2% agarose electrophoresis and **B,** SYBR Green fluorescence melt curve analysis.

(Fig. 6B). This concentration had only a slight effect on the Ct generated by the low concentration of *C. michiganensis* subsp. *sepedonicus* target and no effect on the Ct generated with 5×10^4 cells/ml *C. michiganensis* subsp. *sepedonicus* (Fig. 6B). The addition of 12.5 mM guanidine hydrochloride was sufficient to completely inhibit pCmsC4 RC amplification (Fig. 6B). This concentration resulted in some inhibition at 5×10^3 cells/ml, but had no detectable effect on amplification at 5×10^4 cells/ml (Fig. 6B). To completely abolish *C. michiganensis* subsp. *sepedonicus* detection at 5×10^3 cells/ml and 5×10^4 cells/ml, 15 mM and 25 mM guanidine hydrochloride were required, respectively.

These experiments demonstrated that the two different inhibitors affected the Ct of the pCmsC4 reaction control at concentrations lower than those required to observe the same effect on the Ct of the *C. michiganensis* subsp. *sepedonicus* target. Inhibition is, therefore, unlikely to produce a false negative result for *C. michiganensis* subsp. *sepedonicus* without either abolishing pCmsC4 amplification, or at least increasing the Ct generated in the FAM channel by pCmsC4.

Determination of a quantitative threshold for inhibitor detection. For the validation of negative results in routine analysis, a threshold for the maximum acceptable Ct generated by the pCmsC4 reaction control had to be established. The FAM channel Cts produced by the *C. michiganensis* subsp. *sepedonicus*–negative samples were plotted as a frequency histogram (Fig. 7). The data approximated a normal distribution ($P = 0.067$), with a mean Ct of 31.33 and a standard deviation of 0.65. Three Grubbs' outliers ($P < 0.05$) were detected with Cts 33.41, 35.07, and 37.04 (Fig. 7). The other Cts obtained ranged between 29.44 and 32.34—wider than expected considering that each Ct was generated with the same concentration of pCmsC4 template. This observed range was skewed toward the lower Cts, and may have been the consequence of products amplified in spurious, nonspecific side reactions. This would have contributed to an increase in the overall SYBR Green fluorescence in some of the samples, thus lowering the Ct value obtained.

The maximum Ct acceptable for validation of negative results was set as the value that would likely be greater than 99% of all Cts generated by pCmsC4 in negative samples in the absence of inhibitors. In other words, 99% of Ct values obtained in valid reactions generating negative results for *C. michiganensis* subsp. *sepedonicus* would likely be less than this threshold. Since 99% of a one-tailed Gaussian distribution have values less than 2.326 standard deviations above the mean, the maximum acceptable Ct value was the mean (31.33) plus 2.326 times the standard deviation (0.65); or 32.84. Valid negative results, therefore, would have to generate Ct values less than 32.84.

Performance with diagnostic samples. In addition to the samples previously examined (Table 3), another set of 234 diagnostic samples was analyzed using the TaqMan assay. Of these, 71 were first used to compare fluorescence signal in real-time PCR with visualization of amplicons by agarose electrophoresis as in conventional PCR. Out of 28 of these samples that generated a Cy5 signal, all but 2 generated an 85.5°C melt peak, and all but one generated a 152-bp band in agarose electrophoresis. For one of the inconsistent samples, only the 94.5°C melt peak was observed even though both bands were visible in agarose electrophoresis, while the other inconsistent sample had a very high Ct of 39.73 in the Cy5 channel, no melt peak, and the 152-bp band was not visible in agarose electrophoresis. These two samples might be considered weak or unconfirmed positives, as the identity of the fluorescence signal could not be confirmed by melt peak analysis. Overall, however, the results show that the fluorescence signal plus melt peak analysis of the PCR results correlated very well with the result obtained by agarose electrophoresis (Fig. 8).

The remaining 43 samples of this group tested negative in PCR and did not yield the 152-bp amplicon in agarose electrophoresis. All except five of these samples had FAM Cts lower than the upper threshold of 32.84, melt peaks within the acceptable range of 94.20 to 94.86°C, and the 242-bp reaction control amplicon was visualized in agarose electrophoresis (Fig. 8). Four of the five inconsistent samples had melt peaks outside the acceptable range but displayed the 242-bp band, suggesting that the acceptable melt peak temperature range may be overly stringent. One sample did not produce an FAM signal and only a weak 242-bp band in agarose electrophoresis, which suggested the presence of inhibitory substances.

The serological test results for the entire 234 sample set were compared with the results obtained in the TaqMan PCR assay (Fig. 9). All 13 samples in this data set that were clearly positive by ELISA and IMF were also clearly positive in the real-time TaqMan PCR assay. However, of the 96 samples that were ELISA-positive but IMF-negative (i.e., with <150 fluorescing cells/30 microscope fields), slightly less than half (47 samples) were positive by PCR and were confirmed with the appropriate melting peak. Forty of these 96 samples were negative in PCR and confirmed as such by the Ct and melting peak of the internal reaction control. Another

Table 3. Effect of pCmsC4 (100 copies/reaction) on the specificity of the TaqMan detection method for *Clavibacter michiganensis* subsp. *sepedonicus*

	Number tested	Positive results			Negative results		
		Total	Confirmed[a]	152 bp[b]	Total	Validated[c]	Confirmed[a]
Positive samples[d]	28	28	27	28	0	n/a[e]	n/a
Negative samples[f]	50	0	n/a	n/a	50	47	47

[a] Result confirmed with melt temperature of amplification product 85.1 to 85.9°C for positive results and 94.20 to 94.86°C for negative results.
[b] 152 bp refers to molecular size of amplicon generated by the *Clavibacter michiganensis* subsp. *sepedonicus* target.
[c] Result validated with the FAM Channel cycle threshold (Ct) ≤ 32.84.
[d] Samples had previously tested positive in enzyme-linked immunosorbent assay (ELISA) and immunofluorescence microscopy.
[e] Not applicable.
[f] Samples had been sourced from farm units with no prior history of *Clavibacter michiganensis* subsp. *sepedonicus* infection; tested negative in ELISA.

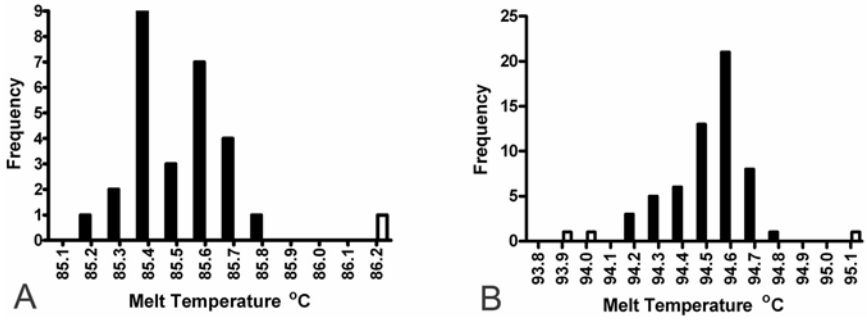

Fig. 5. Frequency histograms of melt peak temperatures of amplicons generated with the *Clavibacter michiganensis* subsp. *sepedonicus* primary target ($n = 28$; **A**) and the pCmsC4 reaction control ($n = 60$; **B**) template DNAs. Measurements falling within the normal distribution of each data set are displayed as black bars, while Grubbs' outliers ($P < 0.05$) are displayed as white bars.

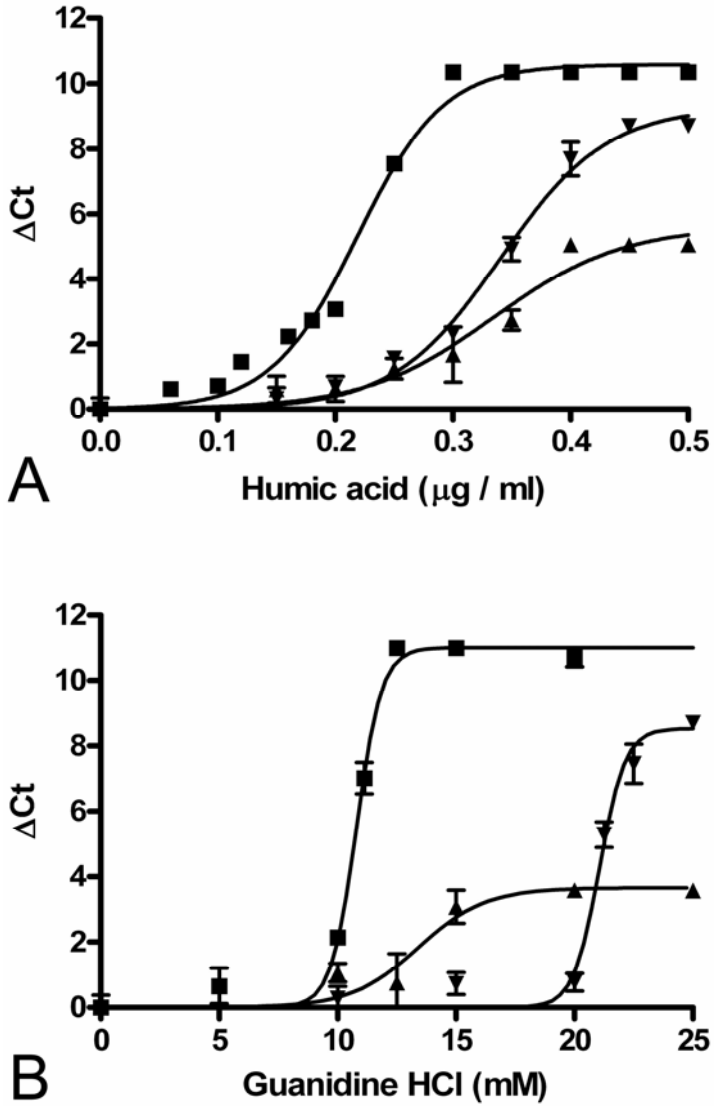

Fig. 6. Effect of humic acid **(A)** and guanidine hydrochloride **(B)** concentration on the change in cycle threshold (Ct) generated by the pCmsC4 reaction control (■) and the Cts generated by 5×10^4 (▼) and 5×10^3 (▲) CFU of *Clavibacter michiganensis* subsp. *sepedonicus* per ml of homogenate. Error bars represent the standard deviation around the mean of triplicate measurements.

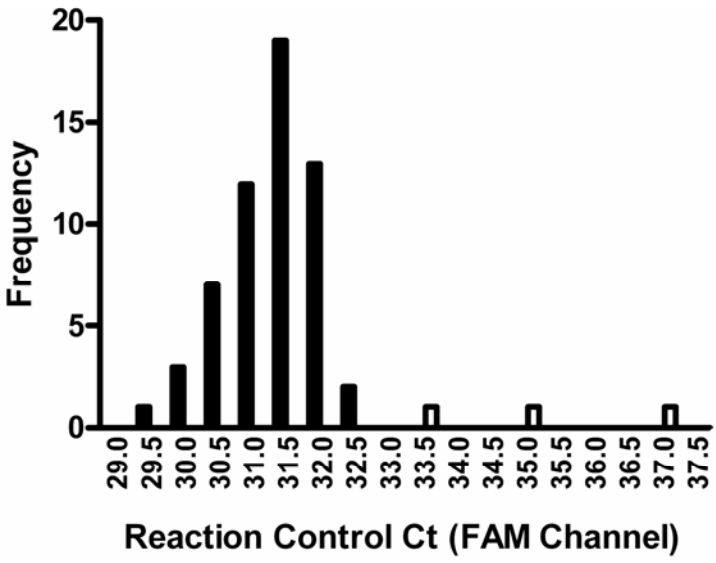

Fig. 7. Frequency histogram of FAM Channel cycle thresholds (Cts) generated by pCmsC4 in known negative samples ($n = 60$) with the TaqMan assay. Black bars represent data fitting a Gaussian distribution. White bars represent Grubbs' outliers ($P < 0.05$).

four ELISA-positive but IMF-negative samples also produced a Cy5 signal but produced inadequate amplicon to permit melt peak analysis. The latter results suggest a weak positive result, as confirmation was not achieved. In addition, 5 samples with no signal in the Cy5 channel generated elevated Cts in the FAM channel, indicating an invalid negative result. These 5 samples were flagged as potentially containing PCR inhibitors.

Of the 125 ELISA-negative samples in this data set, 88 samples were negative by PCR, and the absence of inhibitors was confirmed by the FAM signal and melt peak of the reaction control amplicon. In another 12 samples, the Ct of the FAM signal was either higher than the maximum acceptable Ct value that had been set (9 samples) or did not generate melting peaks within the acceptable melt parameters (3 samples), thus generating an invalid negative result. The 9 samples were also flagged as containing PCR-inhibiting substances. Twenty-five of the 125 ELISA-negative samples were positive in the PCR test for *C. michiganensis* subsp. *sepedonicus* and confirmed by melt peak analysis for 20 of them (Fig. 9). The 5 samples that could not be confirmed by melt peak analysis produced inadequate amplicon for the analysis, probably because of a low amount of template DNA from low level infections with *C. michiganensis* subsp. *sepedonicus*.

The 14 samples that generated invalid negative results, and were flagged as containing PCR-inhibiting substances, were re-extracted to further purify the DNA. A second PCR test on 13 of these samples yielded the expected reaction control fluorescence signal and melt peak confirmation. The 14th sample, however, required a further 1:10 dilution to obtain a reaction control signal. This sample was also confirmed negative.

It should be noted that the samples in this data set do not represent the overall population because the lots were not sampled randomly. While some of the samples were from production units that have never had ring rot, the majority were selected from lots requiring supplementary testing because of their association with a ring rot positive farm unit, or had generated inconclusive results upon initial testing by the standard method. While 47 more samples were found to be positive by the real-time TaqMan PCR procedure than by the serological procedures for this particular sample set, many of the ELISA-positive, IMF-negative samples did contain fluorescing cells. These were rated negative for IMF because the numbers of fluorescing cells were below the established threshold (4). In routine testing, detecting fluorescing cells below the established threshold would have triggered additional testing and possibly re-sampling to avoid false positive diagnoses and their associated regulatory

and financial impacts. The greater sensitivity attained by PCR compared with the serological test is apparent from the 8.5% of samples in this sample set that were ELISA-negative but PCR-positive. Nevertheless, this was likely an overestimation of the actual proportion of positive samples that are typically undetected in ELISA because of the nonrandom selection of the sample set.

The ELISA method is well suited to large-scale screening. It is economical, simple to perform, and facilitates a high sample throughput. While less sensitive than PCR, the results of this study have suggested that only a small percentage of positive samples are undetected in ELISA, even in a positive-biased population. In seed potato certification, the impact of these potentially undetected samples can be offset by utilizing strategic sampling regimes, and targeting higher risk areas and suspect tuber lots with more intensive sampling and screening. Unless it becomes economically feasible, it is unlikely that PCR will replace ELISA as the primary screening method for C. *michiganensis* subsp. *sepedonicus* infection in seed potatoes in the near future. This PCR method does have considerable promise, however, as a confirmatory method to replace or supplement IMF.

In summary, it is concluded that including an internal reaction control in the real-time PCR TaqMan assay for the detection of *C. michiganensis* subsp. *sepedonicus* provides a means of detecting false negative results that may arise as a consequence of reaction failure. Our reaction control consisted of a sequence unrelated to the primary *C. michiganensis* subsp. *sepedonicus* target, flanked by primer sequences designed to specifically detect *C. michiganensis* subsp. *sepedonicus* in a TaqMan assay. This type of reaction control has been used previously in the conventional PCR methods for the detection of *Erwinia carotovora* subsp. *atroseptica* (13), *Agrobacterium tumefaciens* (3), and *C. michiganensis* subsp. *sepedonicus* (12).

In a PCR reaction, the inclusion of a secondary DNA sequence containing the same primer binding sites as the primary target could potentially create unwanted competition for the primers and other reaction components, and thereby reduce the sensitivity of the PCR method for the desired primary target. In addition, nonspecific side reactions may produce false results, or further reduce the efficiency of the primary reaction. It was demonstrated, however, that the reaction control pCmsC4, used at 100 copies/reaction, had no deleterious effects on either the sensitivity or the specificity of the TaqMan assay. The amplification efficiency of the reaction control was lower than that of the primary target. This was probably attributable to the high GC content of the reaction control, and indicated that the amplification of the reaction control would be very unlikely to compete effectively with the amplification of the primary diagnostic target.

Finally, by setting specific Ct and melt peak criteria as positive/negative thresholds for detection of the *C. michiganensis* subsp. *sepedonicus* target and the reaction control, test results could be objectively categorized as confirmed positive, confirmed negative, or requiring additional work. Obviating the need for subjective interpretation of test results by setting of specific threshold values is particularly important for diagnostic laboratories that operate within a quality assurance program.

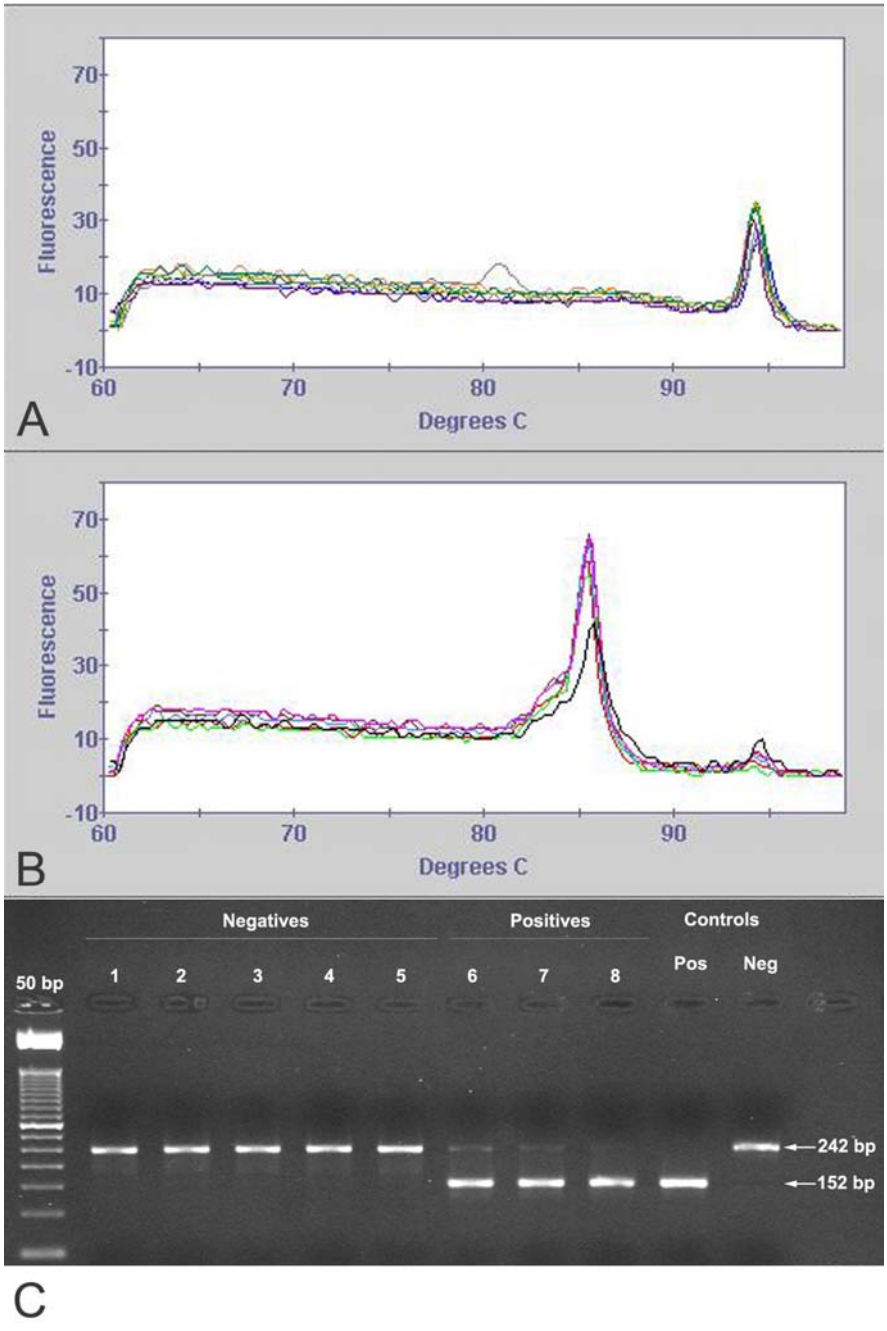

Fig. 8. Typical melting profiles of negative **(A)** and positive **(B)** diagnostic samples, and their correlation with the appearance of 242- and 152-bp bands in agarose electrophoresis, respectively **(C)**. **C,** 50 bp, 50-bp ladder molecular size standard; lanes 1 to 5, negative samples generating a 242-bp amplicon and a ca. 94.5°C melting peak **(A)**; lanes 6 to 8, positive samples generating a 152-bp amplicon and a ca. 85.5°C melt peak **(B)**. Pos, positive control; Neg, negative control.

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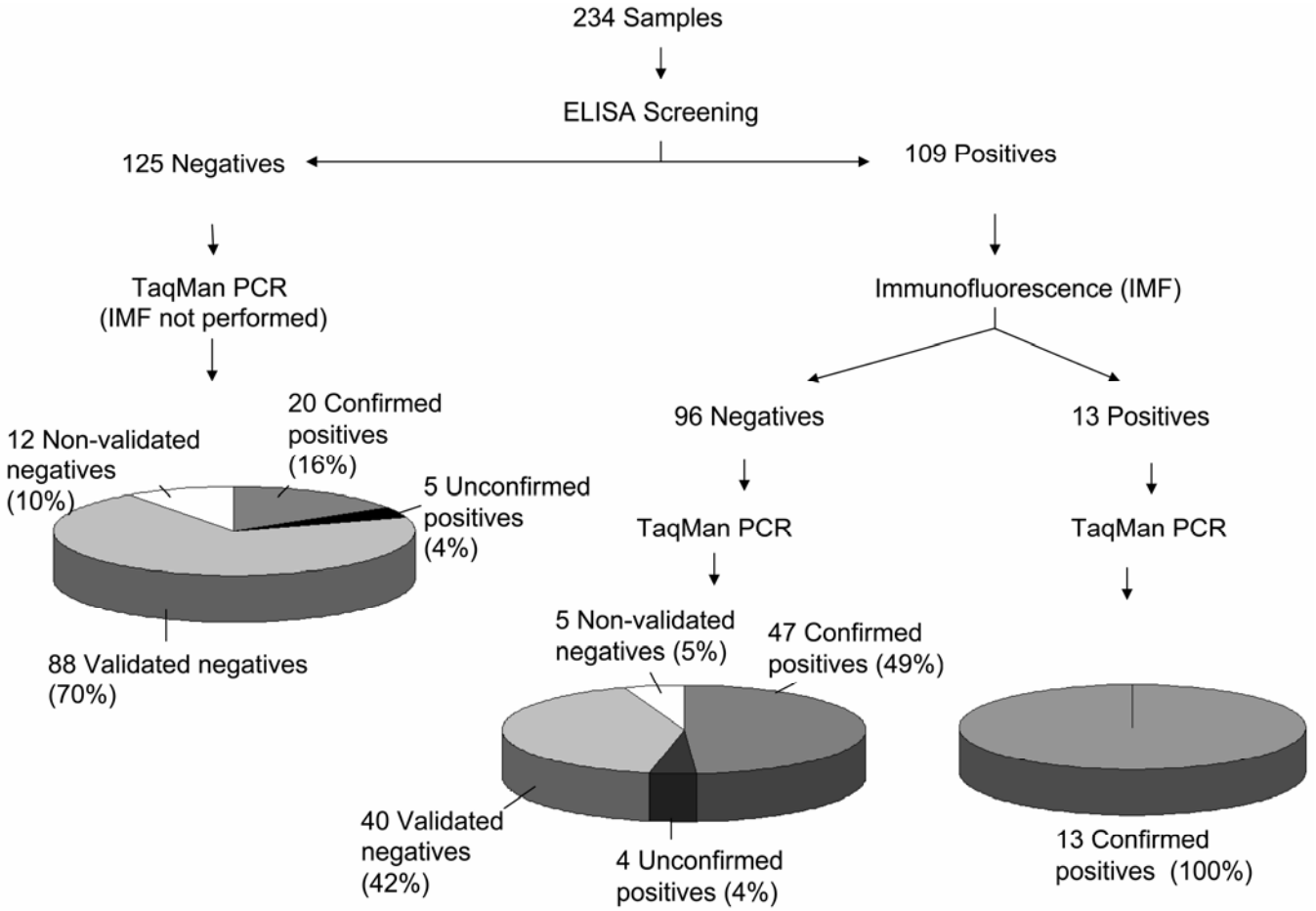

Fig. 9. Comparison of real-time polymerase chain reaction (PCR) (TaqMan assay) and serological results generated for 234 diagnostic samples tested for *Clavibacter michiganensis* subsp. *sepedonicus*. Confirmed positives were those that generated a signal in the Cy5 channel and a melt peak between 85.11 and 85.89°C. Unconfirmed positives were those generating a signal in the Cy5 channel, but failing to generate a melt peak between 85.11 and 85.89°C. Validated negatives were those generating no signal in the Cy5 channel, with a cycle threshold (Ct) in the FAM Channel less than 32.84 and a melt peak between 94.20 and 94.86°C. Nonvalidated negatives either failed to generate an FAM Channel Ct less than 32.84 or failed to generate a melt peak between 94.20 and 94.86°C.

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