



Correlation of PCR and IFAS with eggplant bioassays for identification of *Clavibacter michiganensis* subsp. *sepedonicus*

Ahmed L. Abdel-Mawgood^{1,3*} and Thomas L. German²

¹Faculty of Agriculture, Minia University, Minia 61111, Egypt. ²Entomology Department, University of Wisconsin-Madison, 1630 Linden Drive, Madison WI 53706. ³Current address: Faculty of Food and Agricultural Sciences, King Saud University, P.O. Box 2460, Riyadh, 11451, Kingdom of Saudi Arabia *e-mail:mawgood9@yahoo.com.

Received 8 January 2006, accepted 19 March 2006.

Abstract

Correlation was examined among immunofluorescence, eggplant pathogenicity bioassays and a PCR assay for identification of *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*) using the novel primer pair *Cms A/Cms B* designed based upon the sequence of the 16S rRNA gene. These primers identified 11 *Cms* isolates in eggplant extracts and an additional 14 isolates in pure culture, and results of PCR using these primers correlated 100% with results of IFAS and eggplant pathogenicity assays. *Cmm*, *Clavibacter michiganensis* subsp. *michiganensis*, did not produce symptoms in eggplant and was not identified by IFAS or PCR. These results demonstrate that PCR with the primer pair *Cms A/Cms B* can be as a complimentary test to IFAS and eggplant pathogenicity assays for the detection of *Cms* isolates found in the Midwestern United States.

Key words: Bacterial ring rot, seed potato certification, PCR, IFAS, eggplant bioassay, *Cms*, *Corynebacterium sepedonicum*, *Clavibacter*, *Cmm*, potato pathogen, 16s rRNA gene.

Introduction

Clavibacter michiganensis subsp. *sepedonicus* (*Cms*)⁵ formerly known as *Corynebacterium sepedonicum* ([Spieck. & Kotth.] Skapt. & Burkh.) is the causal agent of ring rot of potato. Symptoms of bacterial ring rot infection appear late in the growing season if at all⁸, and the bacterial pathogen is carried in the seed tuber and is easily mechanically transmitted. Infected tubers serve as a source of inoculum for uninfected tubers during handling, planting and harvesting. Consequently, ring rot is a disease of quarantine significance and many potato seed programs have adopted a “zero tolerance” policy toward the pathogen. Hence, early detection of the *Clavibacter* infection is important, especially in seed potato fields.

Several assays have been developed to detect *Clavibacter* in field material. The eggplant (*Solanum melongena* L.) assay is specific and sensitive, but requires a long incubation time from inoculation to symptom development². ELISA (enzyme-linked immunosorbent assay) using monoclonal antibody 1H3 is easy and inexpensive, but detects only the mucoid strains⁷. More recently, monoclonal antibodies Mn-Cs1 were developed that can distinguish between the *Cms* and other related species, however, no available data on ability to detect various isolates¹⁵.

Previously developed probes for specific hybridization have been shown to provide a sensitive detection method²⁴, but the use of radioactive materials requires extensive training and is too impractical and expensive for routine use in screening procedures. IFAS (immunofluorescent antibody staining) using monoclonal antibody 9A1 is more time consuming and requires more training than ELISA⁶, but is an accepted and successful detection method.

PCR (polymerase chain reaction) techniques have been shown to be sensitive^{12,14}, specific for *Cms*¹⁰ and can be used for routine

testing²⁸. Several PCR-based assays for detection of *Cms* were developed. These assays used different techniques to generate primers specific for *Cms* detection. These can be summarized as follows: (1) 16S rRNA gene sequences as a target for specific PCR for detection purposes^{14,16}, (2) new procedure named TP-RAPD based on DNA fingerprints²⁰, (3) PCR based on repetitive sequence using the BOX-AIR primers²⁵, (4) PCR based on an RNA intergenic region¹¹, (5) RAPD based assay using 30 synthetic decamers²² and finally (6) nested PCR using primers derived from the sequences of 16S rRNA gene and insertion element IS1121⁹. Moreover, PCR was used for differentiation between the different subspecies of *Clavibacter michiganensis*^{13,17,18}.

Several methods for the detection of the products were also used in addition to agarose gel-based method, TaqMan technique based on automated real time PCR²³; a technique named AmpliDet RNA, based on molecular beacon and automated PCR machine²⁶, and plate capture assay¹.

Comparisons were made between the different methods of detection¹⁹. Dig-labeled PCR was found to be more sensitive than ELISA in diagnosis of symptomless field potato tubers¹². PCR, ELISA and DNA hybridization had 36.2, 35.8 and 29.1% efficiency, respectively²⁴. PCR, bioassay and immunofluorescence microscopy, all have similar sensitivity and are better than fluorescent *in situ* hybridization²¹.

Due to the potential economic impact of a bacterial ring rot diagnosis to seed growers, it is the widespread view in the seed potato certification industry that reliance on the results of one test alone should not be used to diagnose bacterial ring rot in a seed lot. Thus, correlation among results of different tests needs

to be thoroughly examined, particularly among biological, serological and molecular tests. When PCR was compared to IFAS and eggplant bioassays as standards for comparison ¹⁶, 100% correlation among these tests was not observed, possibly due to detection of non-pathogenic or non-viable cells, and a strict threshold for IFAS positives.

In this report, we describe a PCR assay with 100% correlation with IFAS identifying *Cms* isolates causing characteristic bacterial ring rot symptoms in eggplant.

Materials and Methods

Bacterial isolates: The names and sources of the isolates used in this study are shown in Table 1.

Table 1. Source and geographical origin of *Clavibacter* isolates used in this study.

Isolate	Source	Geographical origin
<i>Cmm</i>	Slack	USA
2 (INM)	Gudmestad	Idaho, USA
3 (OFF)	Gudmestad	North Dakota, USA
4 (SD-1)	Gudmestad	South Dakota, USA
5	Clarke	Idaho, USA
6	Clarke	Idaho, USA
7	Clarke	Idaho, USA
8 (SS-34)	Slack	USA
9	Chaudoir	USA
10	Chaudoir	Minnesota, USA
11	Chaudoir	USA
12	Chaudoir	Wisconsin, USA
20	Slack	Wisconsin, USA
29	Slack	New Brunswick, Canada
34	Slack	Alberta, Canada
44	Slack	Wisconsin, USA
45	Slack	Wisconsin, USA
46	Slack	Wisconsin, USA
48	Slack	Wisconsin, USA
68	Slack	Idaho, USA
73	Slack	North Dakota, USA
92	Slack	Maine, USA
93	Slack	Alaska, USA
94	Slack	Alaska, USA
95	Slack	Alaska, USA
SS13	Slack	California, USA

Confirmation of pathogenicity: *Cms* isolates and *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) were grown on NBY agar medium ²⁷. Suspensions of isolated colonies were prepared in 0.1% proteose peptone No. 3 and 100 µl inoculated into seedlings of 'Black Beauty' eggplant by injection. Seedlings were inoculated immediately after the first set of true leaves emerged. Eggplants were observed for symptoms of *Cms* infection ². Symptomatic tissue was surface sterilized with 70% ethanol and macerated in sterile water with a mortar and pestle. These extracts were plated on NBY agar medium and observed for colony morphology characteristic of *Cms*.

IFAS: The undiluted eggplant extract described above and 1:10 and 1:50 dilutions of this extract in 1X phosphate buffered saline were analyzed via indirect IFAS ⁶ using monoclonal antibody 9A1 (Agdia Inc.). Fluorescein-conjugated anti-mouse IgM (Agdia Inc.) was used as the detection antibody.

10 µl of each extract and dilutions were applied to each well in

12- well teflon coated slides (Erie Scientific) and allowed to dry at room temperature. IFA was performed according to the protocol described in DeBoer ⁶, using 10 µl each of monoclonal antibody and fluorescein-conjugated anti-mouse IgM preparations per well and incubation periods of one hour. Slides were mounted with 2 µl mounting fluid (90 ml glycerol, 10 ml 1X PBS, 0.1 g p-phenylenediamine), covered with a coverslip, and observed using an Olympus microscope with a fluorescein filter.

Eggplant tissue extract that was inoculated with *Cmm* was used as the negative control. A sample was considered positive if it had >150 fluorescing cells with the *Cms* characteristic morphology, wedge-shaped and with dimensions of 0.8–1.2 µm x 0.4–0.6 µm ³, at any dilution in 30 fields.

Design of specific primers for use in PCR: The conserved region of the *Cms* and *Cmm* 16s rRNA genes were amplified using forward (5'-GAGTTTGATCCTGGCTCAG-3' and reverse (5'-GGTACCTGTTACGACTT-3') primers designed for *E. coli* ⁴. The sequences of these amplified fragments were used to design specific *Cms* primers A (5'-CGCACATCTCTGCACGTTTCC-3') and B (5'-CCCCGACTCTGGGATAACTGCTG-3'). These primers are 100% identical to nucleotides 940-960 (primer A) and 99-120 (primer B) of the *Cms* LMG 2889 16S rRNA sequence as reported to GenBank (accession U09764, Li & De Boer ¹⁰).

PCR for identification of *Cms* isolates: PCR was performed on the 1:10 dilution of the eggplant extracts described above, and on isolated colonies of pure bacterial cultures described above. DNA was extracted as follows: 100 µl of the eggplant extract or a single bacterial colony suspended in 100 µl sterile water was boiled for five minutes, and debris was pelleted by centrifugation at 10,000 x G for three minutes. Extract from an uninoculated eggplant leaf and one from an eggplant inoculated with *Cmm* (asymptomatic) were treated in parallel to generate negative controls. Five µl of the supernatant was used as template in a 50 µl PCR reaction containing 100 ng of each primer, 0.25 mM each dATP, dCTP, dGTP and dTTP, 2 mM MgCl₂ and 1U Taq polymerase in 1X reaction buffer (Promega). Thermocycling was as follows: 94°C 2 minutes, followed by 27 cycles of 94°C 1 minute, 63°C 3 minutes, followed by a final step of 60°C 6 minutes. 5 µl of PCR products were analyzed by electrophoresis through 1% agarose and subsequent staining with ethidium bromide.

The fragment was amplified from *Cms* with primers A and B is 820 bp in length. Bacterial cultures were considered positive by PCR if a 820 bp amplicon was present in the PCR products.

Results and Discussion

All *Cms* isolates except SS13 produced definitive symptoms of infection in eggplant (Table 2), including wilting and chlorotic lesions. All of the resulting *Cms* eggplant extractions except SS13 tested positive via IFAS and PCR (Table 2), thus there was perfect correlation between eggplant pathogenicity, PCR and IFAS results. Our inability to identify isolate SS13 as *Cms* could be the result of a low inoculation dose in eggplant, or the result of this isolate not being *Cms*. Uninfected eggplant and *Cmm* were negative by PCR (Fig. 1).

We have demonstrated that IFAS and PCR with the primer pair *Cms* A/*Cms* B could in combination be useful to many Seed Potato Certification programs. Although this study was performed using

Table 2. Comparison of reactions of isolates to PCR, IFAS, and in eggplant.

Isolate	Eggplant reaction	PCR	IFAS
<i>Cmm</i>	-	-	-
2 (INM)	++	+	+
3 (OFF)	++	+	+
4 (SD-1)	+	+	+
5	+	+	+
6	+	+	+
7	++	+	+
8 (SS-34)	++	+	+
9	++	+	+
10	+	+	+
11	++	+	+
12	+	+	+
20	+	+	+
29	+	+	+
34	++	+	+
44	+	+	+
45	++	+	+
46	++	+	+
73	++	+	+
92	++	+	+
48	++	+	+
93	++	+	+
94	++	+	+
95	+	+	+
68	+	+	+
SS13	-	-	-

Reactions of isolates in eggplant: “++” indicates strong symptoms or severe wilting with chlorotic lesions on all or most leaves, “+” indicates moderate symptoms or moderate wilting with localized chlorotic lesions on some leaves, “-” indicates no symptoms. Reactions of eggplant extracts of isolates to indirect IFAS: “+” indicates >150 fluorescing cells in 30 fields in at least one dilution, “-” indicates no fluorescing cells in any fields at any dilution. Reactions of isolates to PCR: “+” indicates presence of the expected amplicon, “-” indicates no product.

pure bacterial cultures and eggplant extracts, both IFAS and PCR can be performed directly on extracts from field materials. The diagnosis of this pathogen is of critical importance to the seed potato certification process because association of *Cms* with a seed lot can lead to de-certification of the infected lot, and in certain situations, de-certification of all lots on a seed farm. Since the economic effects of these actions can be extreme, the decision is often contentious and sometimes results in litigation. As a result, the procedures used to identify *Cms* infection are of critical importance, and sometimes formally called into question. It is therefore important to examine a variety of information involving cultural practices, seed lot data, and laboratory diagnostic procedures to make a determination as to the presence of the pathogen in a seed lot. The data presented here demonstrates that our primer set can be used as an additional tool in the growing arsenal of techniques to insure the integrity of the diagnostic process, providing additional information to be considered in the context of making certification determinations.

Acknowledgments

We are grateful to Professors S. Slack, N. Gudmestad and D. Clarke for providing bacterial isolates and to S.H. De-Boer for critical review of the manuscript.

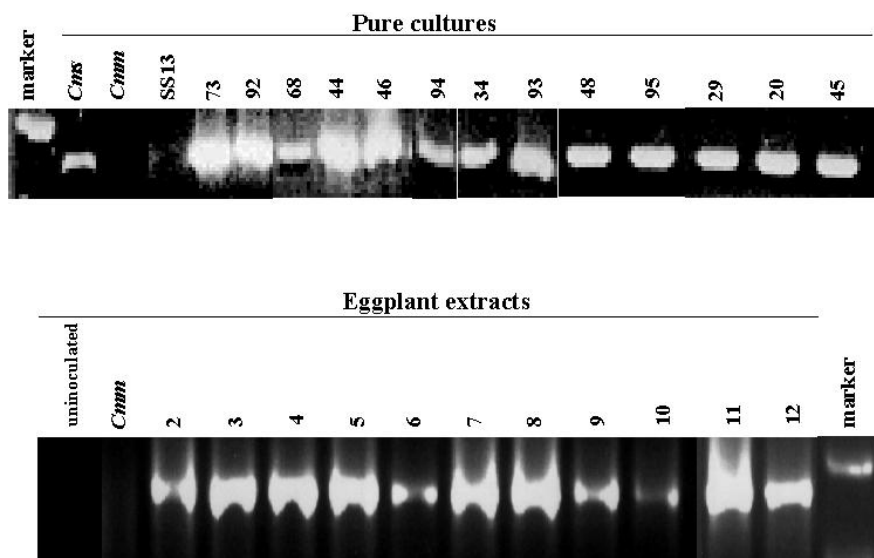


Figure 1. Products of PCR reactions using *Cmm*, leaf sap from uninfected eggplant, eggplant extracts, or *Cms* isolates isolated from eggplant, separated by electrophoresis and visualized by ethidium bromide staining. A standard for size comparison is shown in the far left lane (marker).

References

- ¹Baer, D., Mitzel, E., Pasche, J. and Gudmestad, N. C. 2001. PCR detection of *Clavibacter michiganensis* subsp. *sepedonicus*-infected tuber samples in a plate capture assay. *American Journal of Potato Research* **78**:269-277.
- ²Bishop, A. L. and Slack, S. A. 1987. Effect of inoculum dose, isolate variation and plant growth conditions on the eggplant assay for ring rot. *Amer. Potato J.* **64**:227-234.
- ³Boucher, A. 1994. Protocol for the Detection of *Clavibacter michiganensis* subsp. *sepedonicus* the Bacterial Ring Rot Pathogen of Potato. APHD Official Protocol. Ver. 1.
- ⁴Brosius, J., Dull, T. J., Sleeter, D. D. and Noller, H.F. 1981. Gene organization and primary structure of a ribosomal RNA operon from *E. coli*. *J. Mol. Biol.* **148**:107-127.
- ⁵Davis, M. J., Gillaspie, A. G. Jr., Vidaver, A. K. and Harris, R. W. 1984. *Clavibacter*: A new genus containing some phytopathogenic coryneform bacteria, including *Clavibacter xyli* subsp. *xyli* sp. nov., subsp. nov. and *Clavibacter xyli* subsp. *cynodontis* subsp. nov., pathogens that cause ratoon stunting disease of sugarcane and Bermuda grass stunting disease. *Int. J. Syst. Bacteriol.* **34**: 107-117.
- ⁶DeBoer, S.H. 1990. Immunofluorescence for bacteria. In Hampton, R., Ball, E. and DeBoer, S. (eds). *Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens*. APS Press, St. Paul, MN, pp. 295- 298.
- ⁷DeBoer, S.H., Wiczorek, A. and Kummer, A. 1988. An ELISA test for bacterial ring rot of potato with a new monoclonal antibody. *Plant Disease* **72**:872-878.
- ⁸Dykstra, T. P. 1942. Compilation of results in control of potato ring rot in 1941. *Am. Potato J.* **19**:175-196.
- ⁹Lee, I. M., Bartoszyk, I. M., Gundersen, D. E., Mogen, B. and Davis, R. E. 1997. Nested PCR for ultrasensitive detection of the potato ring rot bacterium, *Clavibacter michiganensis* subsp. *sepedonicus*. *Applied and Environmental Microbiology* **63**:2625-2630.
- ¹⁰Li, X. and De Boer, S.H. 1995. Selection of polymerase chain reaction primers from an RNA intergenic spacer region for specific detection of *Clavibacter michiganensis* subsp. *sepedonicus*. *Phytopathol.* **85**:837-842.
- ¹¹Li, Xiang, Boer, S.H. de, De Boer, S. H. and Li, X. 1995. Selection of polymerase chain reaction primers from an RNA intergenic spacer region for specific detection of *Clavibacter michiganensis* subsp. *sepedonicus*. *Phytopathology* **85**:837-842.
- ¹²Lee, I.M., Lukaesko, L. A. and Maroon, C. J. M. 2001. Comparison of dig-labeled PCR, nested PCR, and ELISA for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* in field grown potatoes. *Plant Disease* **85**:261-266.
- ¹³Louws, F.J., Bell, J., Medina, M., Smart, C.D., Opgenorth, D., Ishimaru, C.A., Hausbeck, M.K., Bruijn, F. J. de, Fulbright, D.W. and de Bruijn, F. J. 1999. Rep-PCR-mediated genomic fingerprinting: a rapid and effective method to identify *Clavibacter michiganensis*. *Phytopathology* **85**:862-868.
- ¹⁴Mills, D., Russell, B.W. and Hanus, J.W. 1997. Specific detection of *Clavibacter michiganensis* subsp. *sepedonicus* by amplification of three unique DNA sequences isolated by subtraction hybridization. *Phytopathology* **87**:853-861.
- ¹⁵Pankova, I. and Kokoskova, B. 2002. Sensitivity and specificity of monoclonal antibody Mn-Cs1 for detection and determination of *Clavibacter michiganensis* subsp. *sepedonicus* the casual agent of bacterial ring rot of potato. *Plant Protection Science* **38**:117-124.
- ¹⁶Pastrik, K. H. 2000. Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato tubers by multiplex PCR with coamplification of host DNA. *Eur. J. Plant Pathol.* **106**:155-165.
- ¹⁷Pastrik, K. H. and Rainey, F. 1997. Differentiation and detection of the subspecies of *Clavibacter michiganensis* by PCR (polymerase chain reaction)-techniques. In Dehne, H.W., Adam, G., Diekmann, M., Frahm, J., Mauler-Machnik A. and Halteren, P. van (eds). *Diagnosis and Identification of Plant Pathogens*. Proceedings 4th International Symposium of the European Foundation for Plant Pathology, Bonn, Germany, 9-12 September 1996. Kluwer Academic Publishers, Dordrecht, Netherlands, pp. 193.
- ¹⁸Pastrik, K. H. and Rainey, F. A. 1999. Identification and differentiation of *Clavibacter michiganensis* subspecies by polymerase chain reaction-based techniques. *Journal of Phytopathology* **147**:687-693.
- ¹⁹Pastuszewska, T., Mierzwa, Z., Lewosz, J. and Burkiewicz, A. 1997. Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato tubers by IFAS and PCR. *Progress in Plant Protection* **37**:336-339.
- ²⁰Rivas, R., Velazquez, E., Palomo, J. L., Mateos, P. F., Garcia, B. P. and Martinez, M. E. 2002. Rapid identification of *Clavibacter michiganensis* subsp. *sepedonicus* using two primers random amplified polymorphic DNA (TP-RAPD) fingerprints. *European J. Plant Pathology* **108**:179-184.
- ²¹Ronda, B.H.N.A.M., Beuningen, A.R. van, Gorkink, R. F. J., Zwaardemaker, N. G., Janse, J. D. and van Beuningen, A.R. 1999. Evaluation of a PCR for detection of *Ralstonia (Pseudomonas) solanacearum* (race 3, biovar 2) and *Clavibacter michiganensis* subsp. *sepedonicus* and comparison with immuno-fluorescence microscopy, plating on semi-selective SMSA medium, pathogenicity test and fluorescent *in-situ* hybridisation. In Proceedings, 51st International Symposium on Crop Protection, Gent, Belgium, 4 May 1999. Part II. Mededelingen Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen, Universiteit Gent **64**:583-591.
- ²²Salava J., Bryxiova, M. and Kokoskova, B. 1998. Identification of plant pathogenic bacteria by random amplified polymorphic DNA (RAPD) assay. *Plant Protection Science* **34**:137-141.
- ²³Schaad, N. W., Berthier-Schaad, Y., Sechler, A. and Knorr, D. 1999. Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato tubers by BIO-PCR and an automated real-time fluorescence detection system. *Plant Disease* **83**:1095-1100.
- ²⁴Slack, S. A., Drennan, J.L., Westra, A.A.G., Gudmestad, N.C. and Oleson, A.E. 1996. Comparison of PCR, ELISA, and DNA hybridization for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* in field-grown potatoes. *Plant Disease* **80**:519-524.
- ²⁵Smith, N. C., Hennessy, J. and Stead, D. E. 2001. Repetitive sequence-derived PCR profiling using the BOX-A1R primer for rapid identification of the plant pathogen *Clavibacter michiganensis* subspecies *sepedonicus*. *European Journal of Plant Pathology* **107**:739-748.
- ²⁶van Beckhoven, J. R. C. M., Stead, D. E. and van der Wolf, J. M. 2002. Detection of *Clavibacter michiganensis* subsp. *sepedonicus* by AmpliDet RNA, a new technology based on real time monitoring of NASBA amplicons with a molecular beacon. *J. Applied Microbiology* **93**:840-849.
- ²⁷Vidaver, A. K. 1967. Synthetic and complex media for rapid detection of fluorescence of phytopathogenic pseudomonas: Effect of the carbon source. *Appl. Microbiol.* **15**:1523-1524.
- ²⁸Zahn, V. 1998. Experiences with the PCR-method in routine testing. *Gesunde Pflanzen* **50**:50-53.