Method for the Detection of *Clavibacter michiganensis* subsp. *michiganensis* on Tomato seed

Crop: Tomato (*Lycopersicon esculentum* L. now *Solanum lycopersicum*)

Pathogen: *Clavibacter michiganensis* subsp. *michiganensis*

Revision history: Version 4, August 2011

Sample and sub-sample size

The recommended minimum sample size is 10,000 seeds, with a maximum sub-sample size of 10,000 seeds.

Note: Sub-samples of 5,000 seeds or larger require an additional concentration step of the extracted bacteria.

Principle

- Extraction from the seed of externally and internally located bacteria
- Isolation of viable *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) bacteria by dilution plating seed extract on two different semi-selective media
- Confirmation of suspect bacterial colonies by morphology on a non-selective medium, followed by a PCR and/or by a pathogenicity assay

Restrictions on use

- This test method is suitable for untreated seed.
- This test method is suitable for seed that has been treated using physical or chemical (acid extraction, calcium or sodium hypochlorite, tri-sodium phosphate, etc.) processes with the aim of disinfestation/disinfection, provided that any residue, if present, does not influence the assay. It is the responsibility of the user to check for such inhibition by analysis, sample spiking, or experimental comparisons.
- The ability to recover Cmm on plates can be influenced by the presence of other microorganisms. A check for such antagonism must be performed by spiking the most concentrated seed extracts with a known number of Cmm-colony forming units (CFUs). See test method description for details on spiking.
- This test method has not been validated for seed treated with protective chemicals or biological substances. If a user chooses to test treated seed using this method, it is the responsibility of the user to determine empirically (through analysis, sample spiking, or experimental comparisons) whether the protective chemicals or biological substances have an effect on the method results.

Note – In this version *Restrictions on Use* has been modified as well as sections 1-3 in *Method Description*. The section *Identification by PCR* is new to this version and the section *Identification by pathogenicity assay* has been revised. New information on media has been added.

Method description

1. Extraction of bacteria from the seed

1.1 Put sub-samples in sterile stomacher bags. Add sterile seed extraction buffer to each bag at a ratio of 4 ml of seed extract buffer to 1 g of seed (v:w). Incubate overnight (minimum 14 hours) at 4°C, and macerate for at least 4 min in a stomacher machine until the extraction buffer has become milky and white particles become visible as a result of endosperm release (see Fig. 1).

Figure 1. The seed extraction buffer and seeds after stomaching

2. Isolation on semi-selective media

2.1. Filter coarse particles from the required volume of extract using a filter bag (Bagfilter® or Bagpage® from Interscience (France) or an extraction bag with synthetic intermediate layer from Bioreba (Switzerland) or Neogen Europe (Scotland)). If a stomacher bag with filter is used this is automatically achieved.

2.2. It is recommended to clear the filtered extract by a low speed centrifugation for 1 min at 1,000 g. This removes fungal spores and facilitates re-suspension of the pellets. Remove the supernatant carefully and use it for further dilution plating.

2.3. Prepare a 10-fold dilution of the seed extract in sterile seed extraction buffer. For sub-samples of 5,000 seeds or larger prepare also a 10-fold concentrated extract. Centrifuge the filtered extract for 5 minutes at 5,000 g. Remove the supernatant carefully and re-suspend the pellet in 1/10 of the original centrifuged volume of sterile seed extraction buffer.

2.4. Spread-plate 0.1 ml of the concentrated (if applicable), the undiluted and diluted extracts onto plates of each of two semi-selective media, CMM1T and either SCM or SCMF.

- It is the responsibility of the user to validate that the increase in colonies in the concentrated extract is in line with the concentration factor. As a guideline there should be consistent 5-fold increase.

2.5. Prepare a 10-fold dilution series of a suspension of a pure culture of a known Cmm reference strain in sterile seed extraction buffer. Spread-plate 0.1 ml of the dilutions on each semi-selective medium. Make sure that at least one plate with 30-300 colonies is obtained on each of the semi-selective media (reference plates for section 2.9).

2.6. As a check on the ability to recover Cmm, spike Cmm into (a separate aliquot of) the most concentrated extract of each subsample at a level of 20 – 50 CFU per 0.1 ml. Spread-plate 0.1 ml of these spiked extracts on a plate of each of the two semi-selective media. Use similarly spiked seed extraction buffer as a control.

- The ratio of spiked Cmm suspension to extract aliquot volume should not be higher than 1:9.
- The suspension for spiking can be made from a fresh Cmm-culture or prepared in advance as a stock in 20% glycerol stored at –80°C. It is recommended to check the concentration of CFUs in glycerol stocks before use.
- In order to minimize chances of cross-contamination it is recommended to separate, in space and/or time, the preparation of the spiking solution and further processing of the spiked samples from the processing of the regular samples.
- Use of an antibiotic resistance marked Cmm-strain is an option to rapidly verify possible false-positives due to cross-contamination.
- Use of a Cmm strain such as *C. m.* subsp. *tessellarius* with a morphology that is distinct from Cmm and from common saprophytes is another option. Such a strain can be spiked directly into the most concentrated seed extracts plated for detection of Cmm. The selected strain must have been shown to be suppressed or overgrown similarly to Cmm on the semi-selective media in the presence of antagonists.

2.7. Incubate plates in the dark at 26-28°C for 10 days. Check plates at 7-10 days.

2.8. Check recovery and morphology of the Cmm reference strain on both media.

2.9. Examine the sample plates for the presence of colonies with typical Cmm morphology by comparing them with the reference Cmm strain. Record the number of suspected colonies as well as other colonies.

- After 10 days of incubation on CMM1T, Cmm colonies are yellow, mucoid and convex (see Fig. 2).
- After 10 days of incubation on SCM, Cmm colonies are translucent grey, mucoid, eventually often irregularly shaped, with internal black flecks (see Fig. 3).
- After 10 days of incubation on SCMF, Cmm colonies are mucoid and translucent grey, often irregularly shaped. The center of the colony can be grey to grey-black depending on the strain (see Fig. 4).
- The colony size and color can differ within a sample. It is particularly influenced by other colonies growing nearby. Isolates may vary in growth speed.

2.10. Examine the plates of the spiked extracts for the presence of colonies with typical Cmm morphology. The test is invalid if such colonies are absent on both media. In this case test a new sample after (further) treatment/disinfestations of the seed lot.

2.11. From sample plates, select, if present, at least 5 suspect colonies per medium per sub-sample for further identification on YDC medium.

3. Identification by morphology on YDC medium

3.1. Transfer selected suspect colonies as well as the reference strains onto YDC medium. Incubate YDC plates at 26 - 28°C for 2-3 days.

3.2. Determine whether transferred colonies have typical Cmm morphology by comparing them with the reference strains and record which of the isolates are still suspected to be Cmm.

- On YDC Cmm is yellow, domed (convex) and mucoid in appearance.

3.3. Identify suspect isolates directly by the pathogenicity assay (section 5) or by a pre-screening using PCR followed by the pathogenicity assay.

4. Identification by PCR

Note: the following instructions on template preparation, PCR conditions and gel electrophoresis are guidelines. The performance of both tests must be verified (see section 5.4.4 in http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2338.2009.02347.x/pdf) using both Cmm and non-Cmm strains. The annealing temperature is a critical variable for optimization. Reaction mixture composition, ramping speed, PCR equipment may interfere with optimum annealing temperature. An annealing temperature gradient must be performed and an annealing temperature must be applied that is between 2 and 3°C below the maximum annealing temperature of the Cmm reference strain.

4.1. Prepare a slightly turbid cell suspension (OD_{600nm} approximately 0.05) in 1 ml sterile distilled water from the suspect cultures on YDC medium and the positive control. Cultures should not be older than 5 days after plating. In addition a non-suspect isolate should be used as a negative control. Heat the suspensions for 10 min at 95–100°C. The suspensions can be stored at -20°C until the PCR analysis.

4.2. **PSA-8/R PCR: gel or SYBR-Green based**

4.2.1. Use the following specific primers (6), where PSA-8 is a modification made to PSA-4 and amplify them to 268 bp.

PSA-8: 5' TTg gTC AAT TCT gTC TCC CTT C 3'
PSA-R: 5' TAC TgA gAT gTT TCA CTT CCC C 3'

4.2.2. Use the following universal bacterial primers to validate the PCR reaction. These primers will give a product of approximately 1500 bp (adapted from 3). Universal bacterial primers that yield shorter products should not be used as they may compete too much with the generation of the specific product.

Bac-8F: 5' gAA gAg TTT gAT CCT ggC TCA g 3'
Bac-1492R: 5' TAC ggC TAC CTT gTT ACg ACT T 3'

4.2.3. Prepare the reaction mixture for gel-based PCR (appendix 1A) or SYBR-Green based PCR (appendix 1B). Carry out PCR reactions in appropriate thin walled PCR tubes in a final volume of 25 µl (20 µl reaction mixture + 5 µl bacterial suspension).

4.2.4. PCR conditions: 5 min incubation at 95°C followed by 40 cycles of 15 s at 95°C, 30 s at 63°C and 90 s at 72°C followed by a final 5 min incubation at 72°C.

4.2.5. Analysis of PCR products:

Gel based PCR - Fractionate 15 to 25 µl of the PCR products by gel electrophoresis during 1.5 h at 150V on a 1.5% agarose gel in 0.5x Tris Borate EDTA (TBE buffer) stained with ethidium bromide. Include a 100 bp ladder.

Analyze the amplification products for a Cmm-specific product of 268 bp and a universal bacterial product of approx. 1500 bp with an ultraviolet transilluminator.

SYBR-Green based PCR - Analyze melting curves of PCR products generated from Cmm and non-Cmm (non-suspect) controls. The dissociation temperature of the universal bacterial product should be approx. 2°C higher than of the Cmm-specific PSA-8/R product.

Analyze the melting curves of suspect colonies for dissociation temperatures specific for Cmm and the universal bacterial product. Allow for a tolerance of ± 0.5°C when comparing dissociation temperatures of controls and suspects.

- Set an appropriate threshold for the Ct value for a negative identification. This is the Ct for a non-Cmm strain obtained with 5-10 x

the amount of DNA per cell needed for a clear positive identification with Cmm. (Note that Cts cannot be directly compared between Cmm and non-Cmm isolates in the multiplex reaction).

4.3. **PTSSK Taqman® PCR**

4.3.1. Use the following specific primers and probe (2). A fragment of 132 bp is amplified.

Primer RZ_ptssk 10: 5' ggg gCC gAA ggT gCT ggT g 3'
Primer RZ_ptssk 11: 5' CgT CgC CCg CCC gCT g 3'
Probe RZ_ptssk 12: 5' FAM-Tgg TCg TCC TCg gCg-MGB-NFQ 3'

4.3.2. Prepare the reaction mixture (appendix 1C). Carry out PCR reactions in a real-time PCR instrument in a final volume of 25 µl (20 µl reaction mixture + 5 µl bacterial suspension). Include at least two positive controls from different bacterial suspensions made according to section 4.1.

4.3.3. PCR conditions: 5 min incubation at 95°C followed by 40 cycles of 15 s at 95°C and 30 s at 60°C. Ramp speed should be 5°C/s.

4.3.4. Determine Ct values; Ct values of positive controls should consistently be lower than 30.

4.4. Combine results of both PCRs (sections 4.2 and 4.3). If both PCRs result in a negative confirmation, the suspect colony is considered not to be Cmm. If one or both PCRs result in a positive confirmation, the pathogenicity assay should be performed to reach a final conclusion about the identity of the suspect colony. Possible PCR outcomes and the conclusions that may be drawn are summarized in the table below.

Table 1: Interpretation of PCR-results

PSA-8/R with internal control SYBR Green or gel-based		PTSSK Taqman	Situation	Conclusion
Internal control	Specific product			
Positive	Negative	Ct > 35 Negative	Expected result for non-Cmm	Negative confirmation, isolate is not Cmm
Negative	Negative	Ct > 35 Negative	PCR inhibition or no template at least in PSA-8/R PCR	No valid result, repeat both PCRs
Either positive or negative, might be suppressed by specific product	Positive	Ct ≤ 35 Positive	Expected result for Cmm	Positive confirmation, perform pathogenicity assay for final conclusion
Either positive or negative, might be suppressed by specific product	Positive	Ct > 35 Negative	Anomalous result but should be considered positive	Positive confirmation, perform pathogenicity assay
Positive	Negative	Ct ≤35 Positive	Anomalous result but should be considered positive	Positive confirmation, perform pathogenicity assay
Negative	Negative	Ct ≤35 Positive	Anomalous result but should be considered positive	Positive confirmation, perform pathogenicity assay

5. Identification by pathogenicity assay

5.1 Grow seedlings of a known susceptible tomato cultivar (e.g. Moneymaker) under suitable conditions until 2-3 true leaves have developed (about 3-4 weeks after sowing).

5.2 Dip a sterile toothpick directly in a suspect colony on YDC medium and inoculate two tomato seedlings by stabbing the toothpick into the stem between the cotyledons and the first true leaf. Use the sharp (rather than blunt) end of the toothpick to avoid excessive damage while introducing inoculum. Include a reference Cmm strain as a positive control and do a mock inoculation with a clean toothpick as a negative control.

5.3 Incubate the inoculated plants at 25-32°C with a minimum of 8 hours daylight.

5.4 Observe the plants for symptoms after 2-3 weeks and compare with the positive and negative control.

- Typical symptoms caused by Cmm are canker formation at the site of inoculation, yellowing and marginal necrosis, and/or wilting of true leaves.

Buffers and media

- Use de-ionized water.
- Autoclave buffers and media at 121°C, 15 psi for 15 minutes.
- Antibiotic (units/mg) activity is critical for the recovery of Cmm. The purity of an antibiotic, and therefore its activity, can vary from batch to batch.
- Antibiotics are not stable in time. Therefore, add antibiotics to the media at a relatively low temperature (< 50°C) and store plates before use in polythene bags at 4°C in the dark. Use plates within a month to maintain the selectivity of the media.

Seed extraction buffer (pH 7.4) per liter

Na_2HPO_4	7.75 g
KH_2PO_4	1.65 g
Tween 20	0.2 ml
$Na_2S_2O_3$ [1]	0.5 g

[1] Recommended when seeds have been treated with hypochlorite

CMM1T (pH 7.7) per liter (1, 5)

Sucrose	10.0 g	
Trizma base (Tris base)*	3.32 g	
TrisHCl*	11.44 g	
$MgSO_4.7H_2O$	0.25 g	
LiCl	5.0 g	
Yeast extract	2.0 g	
NH_4Cl	1.0 g	
Casein hydrolysate	4.0 g	
Agar	15 g	
Polymyxin B sulphate[1]	10 mg	10 mg/ml in distilled water
Nalidixic acid (salt)[1]	28 mg	sodium salt, 10 mg/ml in 0.1 M NaOH
Nystatin[1]	100 mg	100 mg/ml in 50% DMSO/ 50% ethanol

[1] Add after autoclaving

* The pH should be set by using both Tris base and TrisHCl rather than taking either compound and setting with NaOH or HCl which would increase salt concentration. Most importantly this approach secures correct pH without any need for adjusting it when all ingredients have been added. The pH is very critical and most pH meters are less suitable for Tris buffers at high molarity.

SCM (pH 7.3) per liter (4)

Sucrose	10.0 g	
K_2HPO_4	2.0 g	
KH_2PO_4	0.5 g	
$MgSO_4.7H_2O$	0.25 g	
H_3BO_3 (boric acid)	1.5 g	
Yeast extract	0.1 g	
Agar	18 g	
Potassium tellurite[1]	1.0 ml[2]	1% Chapman tellurite solution Difco[2]
Nicotinic acid[1]	100 mg	20 mg/ml in distilled water
Nalidixic acid (salt)[1]	30 mg	sodium salt, 10 mg/ml in 0.1 M NaOH
Nystatin[1]	100 mg	100 mg/ml in 50% DMSO and 50% ethanol

[1] Add after autoclaving

[2] The source of potassium tellurite is critical; the concentration varies depending on the manufacturer. A too high concentration interferes with the recovery of Cmm

SCMF (pH 7.3) per liter (4, 5)

Sucrose	10.0 g	
K_2HPO_4	2.0 g	
KH_2PO_4	0.5 g	
H_3BO_3 (boric acid)	1.5 g	
Yeast extract	2.0 g	
$MgSO_4.7H_2O$	0.25 g	
Agar	18 g	
Potassium tellurite[1]	1.0 ml[2]	1% Chapman tellurite solution Difco[2]
Trimethoprim[1]	80 mg	10 mg/ml in 100% methanol
Nicotinic acid[1]	100 mg	20 mg/ml in distilled water
Nalidixic acid (salt)[1]	20 mg	sodium salt, 10 mg/ml in 0.1 M NaOH
Nystatin[1]	100 mg	100 mg/ml in 50% DMSO and 50% ethanol

[1] Add after autoclaving

[2] The source of potassium tellurite is critical; the concentration varies depending on the manufacturer. A too high concentration interferes with the recovery of Cmm

YDC (Yeast extract - dextrose - $CaCO_3$ Agar) per liter (7, 8)

Yeast extract	10.0 g
D-glucose (dextrose)	20.0 g
$CaCO_3$	20.0 g
Agar	15.0 g

References

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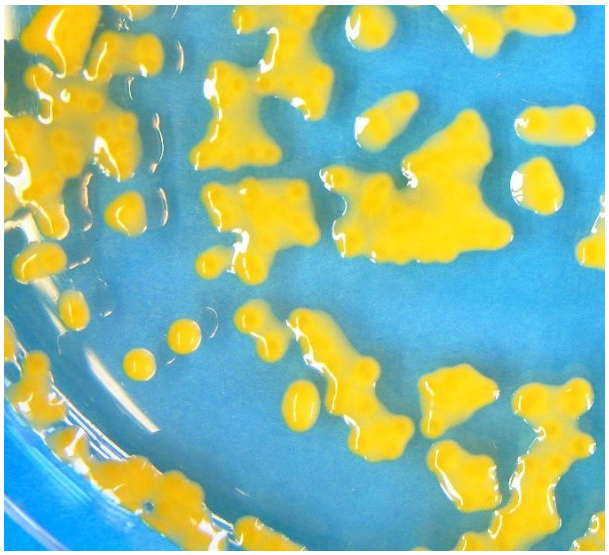

Figure 2. Cmm morphology after 10 days of incubation on CMM1T

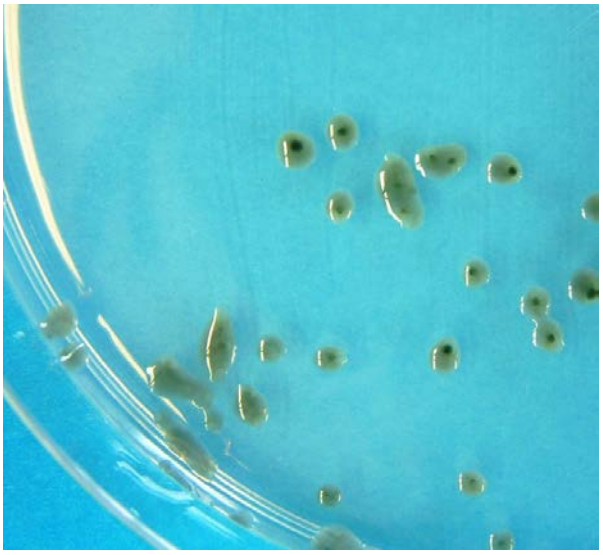

Figure 3. Cmm morphology after 10 days of incubation on SCM

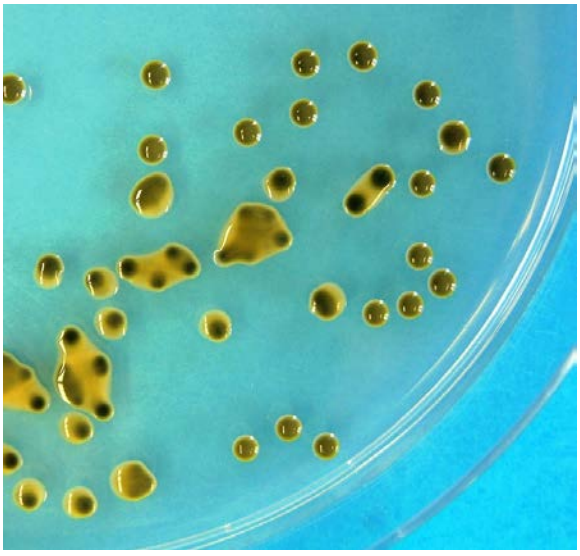

Figure 4. Cmm morphology after 10 days of incubation on SCMF

Appendix 1

A. Preparation of Reaction Mixture for gel-based PCR (section 4.2.3)

Compound	Final concentration	Volume (µl) in 25 µl
Sterile MilliQ water		13.625
10x Buffer*		2.50
$MgCl_2$ (50 mM)	1.5 mM	0.75
dNTP's (10 mM total, 2.5 mM each)	100 µM each	1.00
Primer PSA-8 (20 pmol/µl)	0.40 µM	0.50
Primer PSA-R (20 pmol/µl)	0.40 µM	0.50
Primer 8 F (20 pmol/µl)	0.40 µM	0.50
Primer BacR (20 pmol/µl)	0.40 µM	0.50
Taq Polymerase (5U/µl)	0.025 U/µl	0.125
Bacterial suspension		5.00

* 10x Buffer: Tris-HCl (pH 9.0) - 750 mM; $(NH_4)_2SO_4$ - 200 mM; Tween 20 - 0.1% (v/v)

B. Preparation of Reaction Mixture for SYBR-Green-based PCR (section 4.2.3)

Compound	Final concentration	Volume (µl) in 25 µl
Sterile MilliQ water		5.50
Master mix (2x)*		12.50
Primer PSA-8 (20 pmol/µl)	0.40 µM	0.50
Primer PSA-R (20 pmol/µl)	0.40 µM	0.50
Primer 8 F (20 pmol/µl)	0.40 µM	0.50
Primer BacR (20 pmol/µl)	0.40 µM	0.50
Bacterial suspension		5.00

* Mix containing buffer, salts, heat stable polymerase and SYBR Green

C. Preparation of Reaction Mixture for TaqMan® PCR (4.3.2)

Compound	Final concentration	Volume (µl) in 25 µl
Sterile Milli Q water		6.60
RZ_ptssk 10 (20 pmol/µl)	0.24 µM	0.30
RZ_ptssk 11 (20 pmol/µl)	0.24 µM	0.30
RZ_ptssk 12 (20 pmol/µl)	0.24 µM	0.30
TaqMan® master mix (2x)		12.50
Bacterial suspension		