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Detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seed by a Seed Extract qPCR (SE-qPCR) pre-screening assay

Validation report, July 2022

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ISHI VALIDATION REPORTS

This ISHI validation study has been conducted to determine the fitness of the described method for its intended purpose according to the ISHI Guidelines for the Validation of Seed Health Methods[1] and followed by an independent review of its outcome.

DISCLAIMER

The ISF cannot guarantee that laboratories following these methods described herewith will obtain similar results. Many factors, such as staff skills, laboratory equipment and conditions, reagents and sampling methods can influence the results. Consequently, in case of any litigation ISF will not accept any liability on the use of these methods.

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SUMMARY

Clavibacter michiganensis subsp. *michiganensis* (Cmm) is the causal agent of wilting in tomato (*Solanum lycopersicum*). Seeds are an important route for the dispersal of this bacterium. The ISHI initiated a project to develop a fast seed extract (SE-) quantitative polymerase chain reaction (qPCR) assay for the detection of Cmm, which can be performed as an optional pre-screening step for dilution plating. The Cmm SE-qPCR assay involves collecting bacteria from tomato seed extract by centrifugation, followed by mechanical cell lysis and extraction of bacterial DNA. Samples are analysed for the presence of Cmm DNA in a multiplex qPCR reaction.

In this validation study, the performance criteria analytical specificity, analytical sensitivity, selectivity, repeatability, reproducibility, and diagnostic performance were validated to determine if the Cmm SE-qPCR assay is fit for the intended purpose of serving as a pre-screening assay prior to dilution plating. In validating the analytical specificity of the Cmm detecting qPCR assays using the PTSSK and MVS21+ primers, the alignment of 56 independent Cmm genome sequences show a 100% match, showing good inclusivity of the assays. Alignment of 59 non-Cmm *Clavibacter* spp. with PTSSK and MVS21+ primer and probe sequences showed the exclusivity requirement is met for both PCRs as well. Measured by the detection of liquid cultured Cmm cells, the analytical sensitivity of SE-qPCR is comparable to that of dilution plating. The LOD at a 100% confidence level is the same for both PCR assays (at 5.4 CFU Cmm per mL non-concentrated seed extract). Cmm is detected in four matrices by SE-qPCR with comparable quantitative results, showing good selectivity of the assay. SE-qPCR results are repeatable, as shown by comparable detection of Cmm between replicate samples with 100% accordance of test results. A comparative test (CT) among seven ISHI labs showed good reproducibility of the SE-qPCR assay. All samples were correctly scored by six labs. One lab wrongfully scored two samples with a low Cmm infection as Cmm negative. Accordance and concordance of CT data for low Cmm infected material was over 90%. Test material with a medium or high Cmm infection level, as well as healthy test material, free from Cmm, was identified by CT participants with 100% accordance and concordance. SE-qPCR diagnostic performance in reference to dilution plating is good. All samples spiked with a single Cmm contaminated seed were both dilution plating and SE-qPCR positive, while samples not spiked were all negative for both assays. This shows that both diagnostic sensitivity and specificity of the SE-qPCR assay are 100%.

In this report, it is confirmed that the Cmm SE-qPCR assay is a suitable pre-screening assay for the ISHI Cmm dilution plating assay, being able to detect a single Cmm contaminated seed in a background of 10,000 healthy seeds. Overall, it is concluded that the ISHI Cmm SE-qPCR assay is fit for its intended purpose.

1. INTRODUCTION

The bacterium *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) is the causal agent of wilting in tomato (*Solanum lycopersicum*) (de León *et al*., 2011; Nandi *et al*., 2018). Cmm is of economic importance because infection can lead to a significant loss in crop yield (Chang *et al*., 1992). Seeds are an important route for the dispersal of Cmm (Fatmi *et al*., 1991).

The current ISHI method for Cmm detection in seed samples of tomato includes dilution plating of seed extract (ISHI method Cmm 4.3.1, 2017). This method is, however, time and resource consuming. ISHI initiated a project to develop a seed extract (SE-) qPCR assay for the detection of Cmm. The SE-qPCR assay allows for the fast screening of tomato seed lots against Cmm contamination. In the process flow for Cmm detection, SE-qPCR can be performed as an optional pre-screening step (Figure 1), whereby a negative SE-qPCR result can be taken as a final test result. Because pathogen detection by Cmm SE-qPCR is an indirect seed health test, incapable of proving the presence of viable bacteria, a positive SE-qPCR result is considered to be suspect and should be followed by a direct testing method for confirmation (ISF-ISHI guidelines, 2018). If a sample is determined qPCR Cmm suspect, the Cmm dilution plating method 4.3.1 must be performed on a new seed sample to reach a conclusion about the sample and the seed lot.

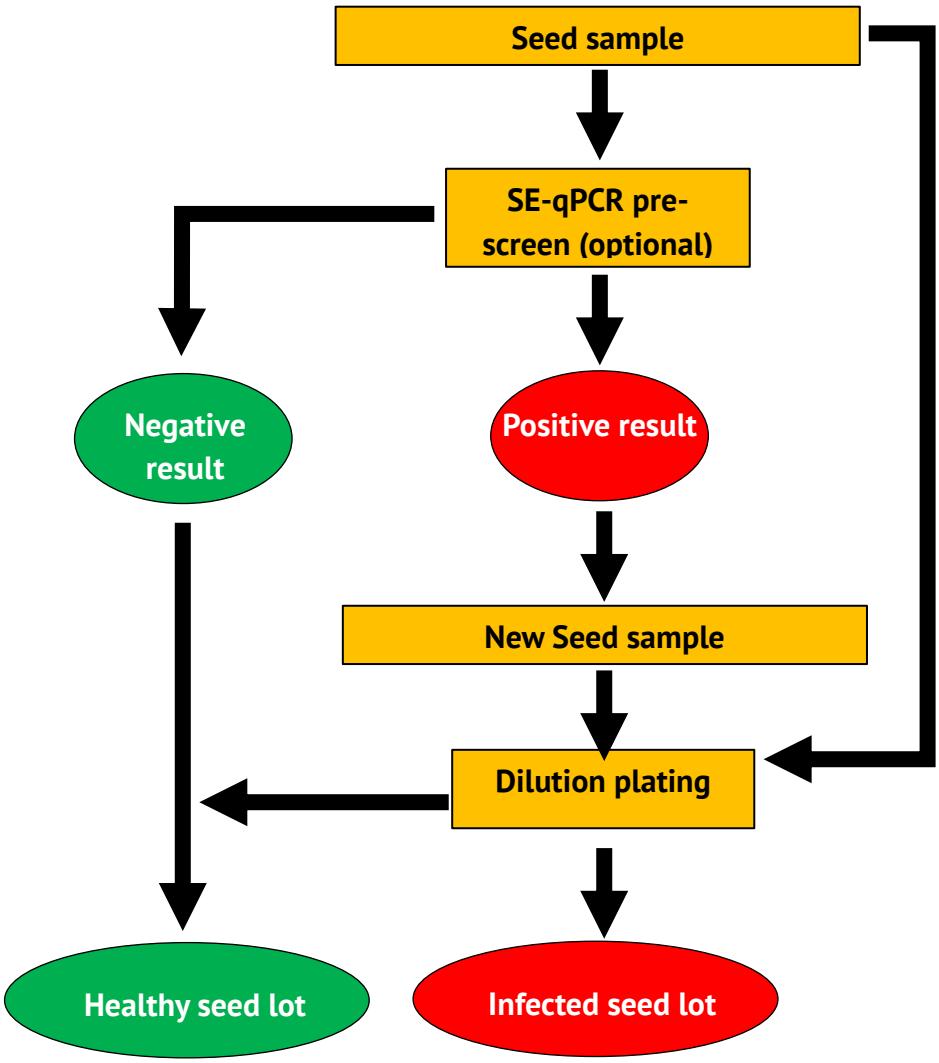

Figure 1. Workflow of the ISHI method for the detection of Cmm in tomato.

The ISHI Cmm SE-qPCR assay (Annex A) is based on the assay developed and validated by Naktuinbouw (Naktuinbouw, 2016a, b). Preparation of the seed extract in the SE-qPCR assay is the same as for the ISHI method Cmm 4.3.1. In the SE-qPCR assay, the seed extract is spiked with a Gram-positive bacterium that mimics the Cmm target, acting as a positive extraction control (PEC). Bacteria are collected from extract by centrifugation and mechanically lysed by bead beating, from which DNA is extracted by commonly available silica gel column purification. A multiplex qPCR reaction is performed on extracted DNA, combining the two Cmm qPCR assays MVS21+ (an adapted version of MVS21; Sudarshana *et al*., 2012) and PTSSK (Berendsen *et al*., 2011) with a qPCR assay to detect the PEC. The PEC also serves as an internal amplification control (IAC) for the PCR (ISF-ISHI Best Practices, 2019).

The ISHI Cmm SE-qPCR assay has been developed using pectinase extracted seeds and pectinase extracted seeds also treated with hydrochloric acid (HCl). HCl treatment meets the standard of an appropriate acid extraction, which is a legally required phytosanitary measure in the EU (EC, 2004). It has been shown previously that SE-qPCR is not compatible with seeds treated with sodium hypochlorite (NaOCl). This is not because of problems with NaOCl residues but because this chemical effectively breaks down DNA, the PCR target. For reference, see Syngenta report 2020a.

2. OBJECTIVES

The work described in this validation report was performed according to the ISHI method validation guidelines (ISF-ISHI guidelines, 2020). The validation work focussed on elements of the ISHI Cmm SE-qPCR assay that are specific for SE-qPCR and that are not already part of the current ISHI Cmm method version 4.3.1. In scope is the validation of the performance of SE-qPCR. Preparation of a seed extract is the same as described in ISHI method Cmm 4.3.1. and is therefore not in scope of the present report.

The SE-qPCR assay must be able to detect Cmm with sufficient sensitivity, independent of the seed background, and in a repeatable and reproducible manner. The performance criteria ***analytical specificity, analytical sensitivity***, ***selectivity, repeatability, reproducibility,*** and ***diagnostic performance*** were validated to show that the Cmm SE-qPCR assay is fit for the intended purpose. In this validation report, besides the results from the experiments, the requirements for each performance criterion are specified and the experimental approach by which each criterion has been validated is described.

Analytical specificity of the two qPCR assays PTSSK and MVS21+ (a slightly modified version of the original MVS21 assay, consisting in an increased melting temperature of one of the primers and the probe by adding a single G-nucleotide, resulting in a more robust qPCR assay) was validated by aligning primer and probe target sequences for Cmm genome sequences (Genbank and previous ISHI sequencing projects) and non-Cmm *Clavibacter* spp. genome sequences (see §3.1). Analytical sensitivity, selectivity and repeatability of the Cmm SE-qPCR assay were validated by spiking liquid cultured Cmm to extracts of multiple healthy seed lots (see §3.2, §3.3 and §3.4, respectively). To validate SE-qPCR reproducibility (§3.5), a comparative test (CT) with seven participating laboratories was organized by Syngenta (for the CT report, see Annex B). SE-qPCR diagnostic performance was validated relative to the ISHI Cmm dilution plating method v4.3.1 by performing both methods on extracts prepared from healthy as well as Cmm infected seed samples (§3.6). Unless indicated otherwise, the SE-qPCR assays and dilution plating were performed without the use of technical replicates. Tables 1, 2 and 3 summarize the experimental approach for SE-qPCR validation.

Table 1. Information summary for validating the ***analytical sensitivity, selectivity,*** and ***repeatability*** of the SE-qPCR assay (§3.2, 3.3 and 3.4).
These three performance characteristics are validated using the same data set generated by Syngenta. A 5-fold dilution series of a fresh Cmm liquid culture was prepared and spiked into the extract of four different healthy seed lots. The dilution series included six concentrations (aiming for 0, 0.4, 2, 10, 50 and 250 CFU/mL non-concentrated seed extract), and each concentration was replicated four times per seed background. Hereby, 16 samples per dilution were tested giving rise to a total number of test samples of 96. All samples were processed by the SE-qPCR assay. In addition, samples were processed according to §2-4 of the ISHI method Cmm v4.3.1, describing the dilution plating (DP) assay.

Seed background	Cmm infection level	#Cmm concentrations	#Replicates	#Samples/ background	Test assays
1.	Healthy	6	4	24	SE-qPCR and DP
2.	Healthy	6	4	24	SE-qPCR and DP
3.	Healthy	6	4	24	SE-qPCR and DP
4.	Healthy	6	4	24	SE-qPCR and DP
			Total #samples	**96**	

Table 2. Information summary for validating the ***reproducibility*** of the SE-qPCR assay (§3.5).
Data is generated by seven ISHI labs in a comparative test (CT, see Annex B). The table summarizes test material for a single lab. The CT organizer provided each lab with material from six different seed backgrounds, each obtained by mixing seed from a healthy seed lot with seed from naturally infected seed lots. For seed mixtures, see Table B1 in comparative test report annex B. Seed backgrounds ranged in the level of Cmm infection. One seed background was healthy, free from Cmm infection (N/A at 40 PCR cycles), two seed backgrounds were low infected (Cq 31-33), two backgrounds were medium infected (Cq 30-31), and one background was highly infected (Cq < 28). Three replicates of 10,000 seeds per seed background were tested. The total number of test samples per lab was 18.

Seed background*	Cmm infection level	Subsamples
A.	No qPCR-signal	3
B.	Low	3
C.	Low	3
D.	Medium	3
E.	Medium	3
F.	High	3
	Total #samples	**18**

*Numbering of seed backgrounds in this table is arbitrary and does not represent the designation of backgrounds in the actual CT.

Table 3. Information summary for validation of the ***diagnostic performance*** of the SE-qPCR assay (§3.6). Data was generated by Syngenta. Individual Cmm infected seeds obtained by peduncle inoculation of tomato plants were spiked to 50 subsamples of a healthy seed lot. Ten subsamples were not spiked (NPCs). The total number of test samples was 60. All samples were processed by SE-qPCR and §2-4 of the ISHI method Cmm 4.3.1 describing the dilution plating (DP) assay.

Peduncle spiked subsamples	Non-spiked subsamples (NPCs)	Total #samples	Test assays
50	10	60	SE-qPCR and DP

3. METHOD VALIDATION

3.1. Analytical specificity

Definition ISHI guidelines: *The ability of an assay to detect the target(s) pathogens (inclusivity) while excluding non-targets (exclusivity)*.

The analytical specificity requirements will be met when:

1. Inclusivity. The requirement for inclusivity is set at 100% for both qPCR assays used to detect Cmm in the SE-qPCR assay (PTSSK and MVS21+, see Annex A). No heterogeneity may be observed in the genomic target sequences of PTSSK and MVS21+ primers and probes of all Cmm strains of which whole genome sequences are available (n ≥ 50). Included strains represent the known overall Cmm sequence diversity.
2. Exclusivity. At least 95% of non-Cmm *Clavibacter* spp. Strains selected (n ≥ 30) give a negative PCR result for both PTSSK and MVS21+ qPCRs and/or can be expected to do so on the basis of their genome sequence when available.

The specificity of a PCR depends on the annealing temperature applied during amplification, as well as the composition of the PCR mix. In the method being validated here, MVS21+ and PTSSK qPCR assays are to be combined and are thus used at identical cycling conditions (see protocol in Annex A). The maximum annealing temperature at which the PTSSK assay still performs well is at least 7 °C higher than for the MVS21+ assay. To ensure specificity and overall performance of the PTSSK assay in tomato seed matrices, the PCRs have to be performed at a relatively high annealing temperature, close to the temperature above which the MVS21+ reaction efficiency becomes lower (Syngenta data, not shown).

The MVS21+ qPCR assay is a slightly modified version of the original MVS21 assay, increasing the melting temperature of one of the primers and the probe by adding a single G-nucleotide (Annex A, Table A.3). These modifications result in a more robust qPCR assay at the relatively high annealing temperature (Syngenta data, not shown). Specificity testing data of PTSSK and the original MVS21 assay produced earlier by Naktuinbouw are summarized and discussed in this validation report, providing additional validation information (for reference, see Naktuinbouw, 2016b). Pertinent raw data on PCR conditions as well as strain identification information may be hard to retrieve and/or be confidential. However, the geographic origin of Cmm has been found not to be linked to genetic diversity, while the latter is considered most relevant and addressed above in the specificity requirement for inclusivity.

Experimental approach

For assessing the inclusivity, PTSSK and MVS21+ qPCR primer and probe target sequences were aligned against Cmm genome sequences in Genbank (n=40) and previous sequencing projects (Genetwister, n=10; Bayer Vegetable Seed USA, n=536).

For assessing the exclusivity, non-Cmm *Clavibacter* sp. genome sequences were blasted for MVS21+ and PTSSK(-like) sequences.

Analytical specificity data from *in vitro* experiments for PTSSK and the original MVS21 assay available from previous work was considered sufficient (Naktuinbouw, 2016b) and was not expanded in current validation work. The MVS21+ reverse primer and probe each only differ from the original MVS21 sequences by a single G-nucleotide (Annex A, Table A.3).

Results

Inclusivity

Since the development and publication of the MVS21 and PTSSK qPCR assays (Sudarshana *et al.* 2012, Berendsen *et al.*, 2011), whole genome sequences of a range of Cmm and non-Cmm *Clavibacter* strains have been published (Zaluga *et al.*, 2014, Thapa *et al.*, 2017, Osdaghi *et al.*, 2020) and submitted to Genbank. To date (March 1, 2022), there are 47 Cmm whole genome (chromosome) sequences, of which two are a duplication of the same strain (LMG7333 = CFBP4999) (see Annex D for an overview of Genbank genome assembly and annotation reports). For the purpose of validation of both qPCR assays, large numbers of collection strains have been screened in *in vitro* experiments previously. Naktuinbouw, 2016b reports a collection screen of 53 Cmm isolates, all of which were detected by both PTSSK and MVS21 qPCR assays (inclusivity 100%). Depending on reagent mix composition and annealing temperature applied, mismatches that could compromise the sensitivity of the PCR could be missed in *in vitro* experiments. *In silico* verification of target sequences will unambiguously show the presence or absence of mismatches. This is important as annealing temperature has been raised from 60 °C for colony identification to 67 °C for the detection of Cmm in the SE-qPCR.

Alignment of the 47 Genbank Cmm genomic sequences with the MVS21+ and PTSSK primer and probe sequences shows a 100 % match for all Cmm genomic sequences in Genbank as well as with another 11 Cmm strains of which genomic sequences had been determined in a genome sequencing project in the Netherlands (Genetwister project) (see also Annex D). Of these 11 Cmm strains sequenced, one (NCPPB382) is also present in Genbank. Taken together, with these 46 unique Genbank genomic sequences and the 10 independent Cmm genome sequences, the inclusivity requirement is met.

Note: Bayer Vegetables Seeds had an additional 536 Cmm genome sequences available for analysis of primer and probe sequences. These sequences originate from Cmm strains isolated over 25 years and at least 21 countries. For PTSSK, the alignment showed a perfect match for all sequences. For MVS21+, two sequences of strains from this Bayer Vegetables Seeds collection showed a single nucleotide polymorphism in one of the primers, a “C” to “A” transversion at position 14 in MVS21+F (Cmm_632) and a “C” to “T” transition at position 7 in MVS21+R (Cmm_190). Only in the latter case, this led to a higher Cq in amplification of the DNA of these strains in PCR (J. Demers, personal communication). This raises some doubts about the genuineness of the observed mutation in Cmm_632. Nevertheless, the PTSSK assay would still allow adequate detection of these two strains.

Exclusivity

In vitro experimental data is available for PTSSK and the original MVS21 qPCR assay. Naktuinbouw, 2016b reports a collection screen of 24 non-Cmm *Clavibacter* isolates, none of which were detected by either assay (exclusivity 100%). With the availability of a growing number of genomic sequences of plant-associated and other bacteria, BLAST searches in databases now provide a powerful tool to screen bacterial DNA detection markers for their exclusivity.

BlastN was used with Cmm MVS21+ and PTSSK amplicon sequences as queries in the nr/nt database and the WGS *Actinobacteria* database (*Clavibacter* belongs to the phylum *Actinobacteria*). Next to the 47 Cmm genome sequences, Genbank currently contains 62 non-Cmm *Clavibacter* genome sequences. Due to duplications and even a triplicate sequence (as indicated in Annex D) the number of independent sequences is 53.

For both amplicons, a 100% match was obtained with sequences of *Clavibacter* strains Z001 and Z002. The meaning of this finding will be discussed below after first having covered the other findings.

For MVS21+, a low e-value was only obtained for all three available *C. michiganensis* subsp. *Sepedonicus* (Cms) sequences as well as a *Clavibacter* strain from an animal fecal sample. All four sequences are identical to each other with the MVS21+ probe sequence being fully conserved. However, of the 21 nucleotides of each of both primers, ten do not match (see figure 2), which will exclude amplification in PCR.

```
CP033724.1   1874111   CTAGTTGCTGAATCCACCCAGCGGAACGGTCTGCCACCCGATGTTGTTGTTCCGGCGATTTCCGAAACGAGAGTCAAGCGGTAC   1874194
AM849034.1   418165    TC..CC.T.GA.G..G….C….................................G..G..AT.CGCG..T..G….A.G.   418082
```

Figure 2. Alignment of the 84 nt MVS21+ amplicon (CP033724.1; Cmm UF1) with sequence AM849034.1 of *C. michiganensis* subs. *Sepedonicus* (Cms) type strain ATCC 33113.

Primer and probe sequences are underlined. Dots indicate identities. The Cms sequence shown and identical sequences of Cms strains CFIA-CsR14 and CFIA-Cs3N, and a strain named Mgla_MAG_34-bin_22 obtained from an animal fecal sample, are the only significantly similar sequences among non-Cmm sequences in Genbank. (Note: the full genome sequence of Mgla_MAG_34-bin_22 also is (nearly) identical to Cms sequence AM849034.1).

The PTSSK amplicon search in the nr/nt database and the WGS database yielded significant hits covering the full amplicon only for strains classified as *Clavibacter*. The presence of the corresponding sequence in non-Cmm *Clavibacter* strains was known at the time the PTSSK qPCR assay was designed (Berendsen *et al*., 2011). This concerned other known subspecies of *C. michiganensis* such as Cms as well as non-Cmm *Clavibacter* strains isolated from tomato seed. Heterogeneity in the sequence was deployed to design PTSSK primers and probe with maximum divergence. DNA from strains representing the known non-Cmm subspecies of Clavibacter and non-Cmm *Clavibacter* strains from tomato seed were also tested in PCR (Berendsen *et al*., 2011, Jacques *et al*., 2012, Zaluga *et al*., 2013). These lab results confirmed the lack of amplification of DNA from non-Cmm *Clavibacter* strains.

Alignment of PTSSK primer and probe sequences with currently available non-Cmm *Clavibacter* sequences (Annex E) reveals at least two mismatches in either both primers or in one primer and its associated probe in most sequences. If this is not the case, as for the three sequences shown in Figure 3, there are three mismatches in one of the primers. Note that lack of amplification has been demonstrated for the minimally deviating Cms PTSSK sequence in the prescribed PCR conditions in the ISHI method for colony identification at an annealing temperature of 60 °C (ISHI Method Cmm 4.3.1 (2017)), whereas in this SE-qPCR protocol 67 °C is applied. For other strains represented in Figure 3, levels of destabilization of primer and/or probe hybridization similar to that for Cms are expected to exclude amplification as well.

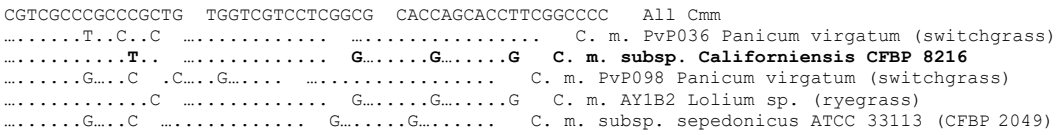

Figure 3. Alignment of PTSSK primers and probe sequences with most similar PTSSK sequences from non-Cmm *Clavibacter* strains.
See Annex E for a complete list. Dots indicate identities. Bold print indicates a strain isolated from tomato.

In 2013, Zaluga *et al.* reported weak signals for several non-pathogenic Naktuinbouw Bacterie Collectie (NBC) strains in a gel-based PCR using the PTSSK primers. The faintness of the bands indicates primer sequence divergence. The higher annealing temperature as well as the inclusion of the MGB probe in the present protocol can be expected to eliminate the unspecific signal in the qPCR assay.

Nine Cmm lookalikes from tomato seed were included in the Genetwister genome sequencing project, of which six can be considered as *Clavibacter* sp. Three have sequences that display a full amplicon sequence for PTSSK (shown in Annex E). All three have primer and probe sequences similar to at least one Genbank entry and have been shown to be negative in *in vitro* PTSSK PCRs.

Clavibacter comparative genomics has revealed a monophyletic origin of Cmm, with at least 99% Average Nucleotide Identity (ANI) (Li *et al.*, 2018, Méndez *et al.*, 2020) between Cmm strains and at most 96% ANI when comparing Cmm strain sequences with those of non-Cmm *Clavibacter* strains. However, two (almost) identical *Clavibacter* sequences in Genbank (of probably clonal strains Z001 and Z002) show an ANI of just below 99% when compared to Cmm sequences (Méndez *et al.*, 2020). The origin of these strains is not traceable in online databases, nor is there any information on pathogenicity for tomato. The absence of certain Cmm specific genes (Thapa *et al.*, 2019, Méndez *et al.*, 2020) in Z001/Z002 genomes could argue in favour of not considering them to be Cmm. However, virulence genes on plasmids and in the genomic pathogenicity island appear to be unstable in some strains of Cmm (Woudt, B., unpublished), which may explain the isolation of pathogenic and non-pathogenic Cmm with identical rep-PCR fingerprint patterns from infected plants (Louws *et al.*, 1998). Note that this explains the choice of ISHI to select the current 'core' genomic markers. The conservation of MVS21+ and PTSSK marker regions in the slightly diverged strain(s) Z001/Z002 supports the inclusivity parameter.

As to the requirement for the exclusivity parameter: with 53 (Genbank) and 6 (Genetwister project) adding up to 59 non-Cmm *Clavibacter* spp. analysed, the exclusivity requirement (n > 30) is met for both PCRs. The additional screening by BLASTing of hundreds of genomes of bacteria in nr/nt and WGS databases did not reveal the presence of homologous sequences in non-*Clavibacter* bacteria either.

Conclusion

Insight into the phylogenetics of strains belonging to the *Clavibacter* species is in line with the conservation of the MVS21+ and PTSSK amplicon sequences in all Cmm strain genome sequences available and the observation of more or less diverged sequences in some related *Clavibacter* (sub)species. Inclusivity is the most important requirement for SE-qPCR, as it is a pre-screen. Only for one or at the most two Cmm isolates out of almost 600 strains analysed, the MVS21+ qPCR assay will be inadequate, which is compensated for by the full conservation of the PTSSK target

region. As to exclusivity, non-pathogenic strains, which would be considered to be Cmm based on their overall genome identity are known to cross-react. As the presence of such strains on tomato seed could well be accompanied by the presence of pathogenic Cmm, their detection in SE-qPCR is desirable. In summary, combining information obtained from the *in silico* analyses and from previously performed *in vitro* experiments, it is concluded that the overall inclusivity and exclusivity of both PTSSK and MVS21+ combined is 100%. The requirements for both inclusivity and exclusivity are met.

3.2. Analytical sensitivity

Definition ISHI guidelines: *Smallest amount of the target pathogen that can be detected i.e. the limit of detection (LOD).*

Since Cmm SE-qPCR is a pre-screen for the dilution plating (DP) assay, SE-qPCR analytical sensitivity is validated in reference to DP. The analytical sensitivity requirements will be met when the LOD of the Cmm SE-qPCR assay is shown to be lower than 10 times the LOD of the DP assay (described in §2-4 of ISHI method Cmm 4.3.1). The required sensitivity is derived from experimental work previously conducted using a draft version of the ISHI Cmm SE-qPCR protocol (Syngenta reports 2020b and 2020c, conducted using the original MVS21 qPCR assay). Provisional data showed that the SE-qPCR LOD in detecting live Cmm cells added as a spike is in proximity of- but does not exactly match the theoretical LOD of dilution plating of 10-20 CFU/mL concentrated seed extract (Syngenta report 2020b; Beugelsdijk *et al.*, 2018). Draft results triggered investigating the composition of viable and non-viable cells on Cmm contaminated seeds, for it should be kept in mind that SE-qPCR and dilution plating are very different assays and a thorough understanding of the assays is needed to compare test results. The SE-qPCR gives information about the amount of template copy numbers, representing DNA of both viable and non-viable bacteria. Dilution plating only gives information about living bacterial cells. Experimental work showed there is an excess of copy numbers originating from non-viable Cmm cells present on seed (>10-fold; Syngenta report, 2020c). Detection of the total Cmm contamination contributes to the analytical sensitivity of SE-qPCR. It is concluded that analytical sensitivity of SE-qPCR is proven equal to DP when the SE-qPCR LOD in detecting liquid cultured (live) Cmm cells does not exceed the LOD of DP by 10-fold, and that hereby SE-qPCR is shown to be fit for purpose in serving as a pre-screen for DP.

Experimental approach

Analytical sensitivity of the ISHI SE-qPCR protocol was determined by spiking seed extract samples with a dilution series of liquid cultured Cmm. The data was generated by Syngenta (ISHI member lab). To make data useful for validating repeatability of the assay as well, the experiment was performed with minimal variations. Table 1 presents an overview of the experiment.

Analytical sensitivity of the SE-qPCR assay was validated using a single dilution series. Independently prepared dilution series were made in previous experiments and contained comparable Cmm concentrations (data not shown). From a logarithmically growing culture of Cmm, a five-fold dilution series comprised of five different concentrations was prepared. It was targeted to add 0.4, 2, 10, 50 and 250 cells per mL of non-concentrated seed extract. Previous data showed that the target concentration range was established with good confidence (Syngenta report 2020b). Cmm CFU concentration of the series was determined by plating 100 µL of each dilution of the series in triplicate on non-selective agar medium. Additionally, to generate

"scoreable" plates for multiple dilutions of the series (20-200 CFUs/plate), each Cmm dilution was diluted further in phosphate buffer (PB) in the same ratio as test samples are spiked, and then plated (Annex A). Plates were incubated at 27 °C and counted after ten days. Plates containing 20-200 CFUs were used to determine the concentration of the dilution series. Results of replicate plates were averaged.

Seed extract was prepared from four different tomato seed batches. Selected seed lots are hydrochloric acid treated and vary in variety and country of origin. One of the batches was an interspecific rootstock of tomato (Table 4)

Table 4. Information on seed lots used in this experiment

Seed background	Health status	Material (variety)	Origin (crop year)
1	Healthy	Rootstock (LYCO AGADIR)	Netherlands (2012
2	Healthy	Seed (DAFNIS)	India (2017)
3	Healthy	Seed (COLBY)	Kenya (2015)
4	Healthy	Seed (DAYLOS-R)	Kenya (2017)

Each concentration of the dilution series was spiked to four replicate samples of extract from each seed lot (after stomaching, before centrifugation, see Annex A). Four extract samples of each seed lot were not spiked with Cmm (negative processing controls; NPCs).

In total, each dilution of the series was tested 16 times, with four replicates in four different seed backgrounds. Each sample was processed by SE-qPCR (according to Annex A) as well as by dilution plating as described in §2-4 of the ISHI method Cmm 4.3.1, enabling direct comparison of the SE-qPCR and dilution plating analytical sensitivities. PCR assays were performed using the 2× PerfeCTa qPCR Toughmix (Quantabio) and CFX Opus 96 PCR machines (Bio-Rad). Data was analysed with CFX Maestro software (Bio-Rad; v2.3)). For all assays, fluorescent thresholds were set at a fixed 200 relative fluorescence units (RFU), and a Cq cut-off value of <40 was applied.

Standard statistical methods were used to determine variation in SE-qPCR and dilution plating data. The LOD of both methods at a detection rate of 100% was determined.

Results

Cmm concentration of the dilution series

The Cmm concentration of the dilution series is shown in Table 5. Calculations are based on the results of plating the series on non-selective media plates (Supplementary Table C1). Results confirm a five-fold concentration difference between dilutions. Plating results for 'dilution' 6 (0 CFU; negative control) were negative.

Table 5. Cmm concentration of the dilution series.
Concentrations correspond to the amount of Cmm spiked per mL of non-concentrated seed extract. See Supplementary Table C1 for raw plating results and calculations.

Cmm dilution	Cmm concentration (CFU/mL non-conc. seed extract)
1	680
2	136
3	27.2
4	5.4
5	1.1
6	0

Dilution plating results test samples

According to the dilution plating section of ISHI method Cmm 4.3.1, seed extract spiked with the Cmm dilution series was plated both non-concentrated and 10-fold concentrated. Raw plating results of the experiment are shown in Supplementary Table C2. Saprophyte count was very low for all samples. All samples spiked with Cmm dilution 6 (0 CFU; negative control) were plating negative. Relative results of samples spiked with the different Cmm dilutions show the expected five-fold difference in Cmm-colony numbers (Figure 4). Also, a 10-fold difference is observed between results of non-concentrated and the 10-fold concentrated samples, as expected. Plating results are comparable between samples spiked with the same Cmm concentration of the dilution series, both between replicate samples of the same seed batch as well as between samples of different batches.

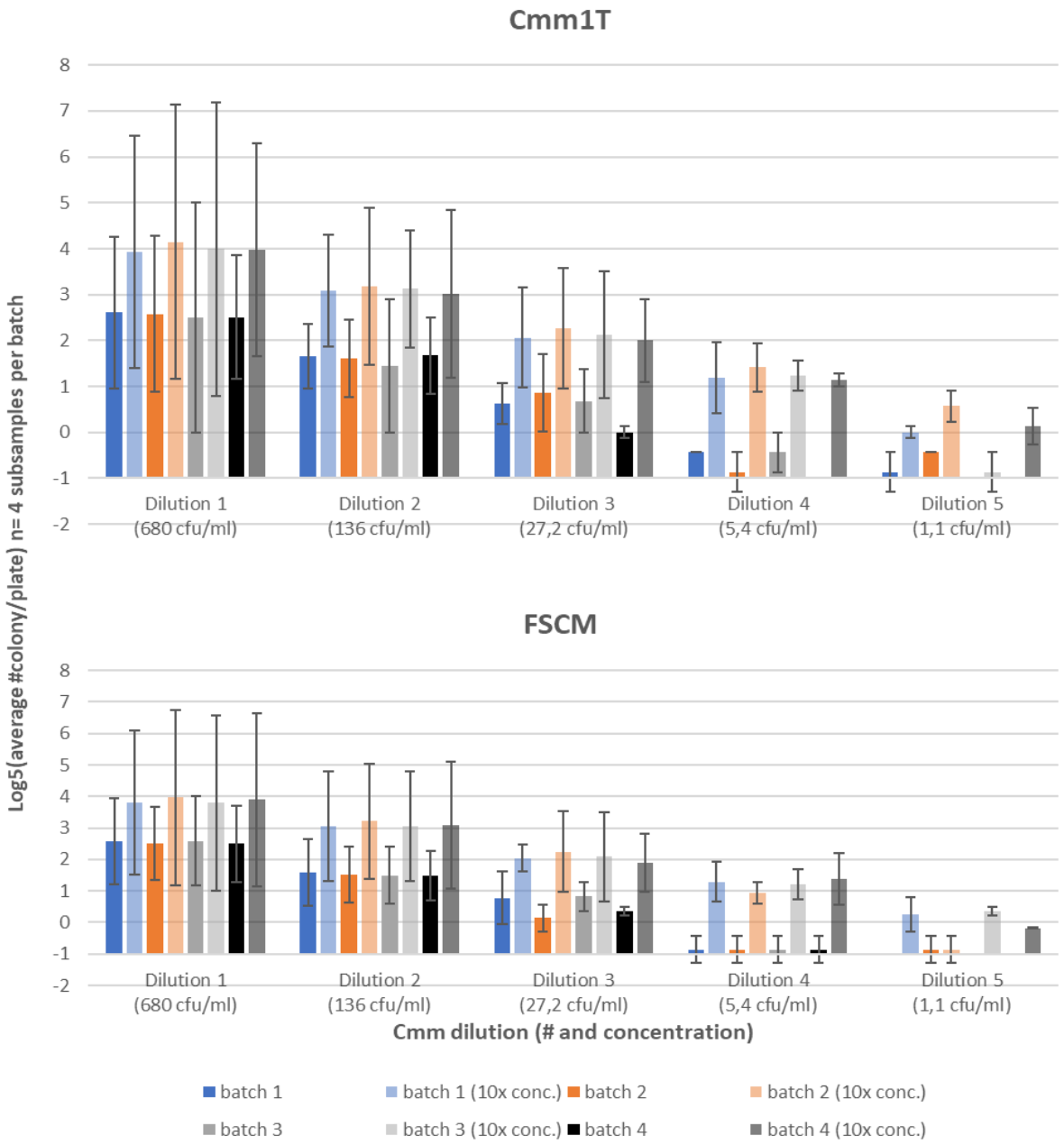

Figure 4. Dilution plating results of test samples.
Extract prepared from four different seed batches was spiked with a five-fold dilution series of liquid cultured Cmm (dilutions 1-5). Extract was plated non-concentrated and 10-fold concentrated on Cmm1T and FSCM media plates. Presented per medium type and per Cmm dilution is the average Log5 (number of colonies per plate) ±Standard deviation of four subsamples tested for each seed batch.

All 16 samples (four batches; four subsamples) spiked with Cmm dilution 4 (5.4 CFU mL^{-1} non-concentrated seed extract) generated dilution plating positive results (Table 6). Cmm dilution 5 (1.1 CFU mL^{-1} non-concentrated seed extract) was detected by plating in 13 out of 16 samples (81.2%). The three subsamples in which Cmm dilution 5 was not detected belonged to different seed batches. The LOD of the Cmm dilution plating assay at a 100% confidence level is 5.4 CFU Cmm per mL non-concentrated seed extract.

Table 6. Dilution plating results of test samples spiked with dilutions 4 and 5 of the Cmm dilution series (respectively 5.4 and 1.1 CFU ml^{-1} non-concentrated seed extract).
Extract prepared from four subsamples of four different seed batches were spiked with each Cmm dilution. The extract was plated non-concentrated, and 10-fold concentrated on Cmm1T and FSCM media plates.

Sample (Seed batch_Cmm dilution,Rep.)	Cmm1T		FSCM	
	Non-conc.	10x conc.	Non-conc.	10x conc.
1_4.1	2	5	0	4
1_4.2	0	8	0	9
1_4.3	0	11	1	9
1_4.4	0	3	0	10
2_4.1	0	8	1	6
2_4.2	1	8	0	3
2_4.3	0	13	0	6
2_4.4	0	10	0	3
3_4.1	0	7	0	6
3_4.2	1	9	1	10
3_4.3	0	5	0	5
3_4.4	1	8	0	7
4_4.1	0	6	1	5
4_4.2	0	5	0	10
4_4.3	0	8	0	8
4_4.4	0	6	0	14
1_5.1	0	2	0	0
1_5.2	0	1	0	5
1_5.3	1	1	0	1
1_5.4	0	0	0	0
2_5.1	0	2	0	0
2_5.2	0	3	0	0
2_5.3	0	2	0	0
2_5.4	2	3	1	1
3_5.1	0	0	0	0
3_5.2	0	0	0	2
3_5.3	0	1	0	3
3_5.4	0	0	0	2
4_5.1	0	0	0	2
4_5.2	0	0	0	0
4_5.3	0	4	0	1
4_5.4	0	1	0	0

SE-qPCR results test samples

Raw PCR results of the experiment are shown in Supplementary Table C3. Results for PCR control samples were as expected (PACs were positive; NTCs N/A). All samples spiked with Cmm dilution 6 (0 CFU; negative control) were PCR negative. The Cmt internal control was detected in all samples with comparable results (average Cq 29.1 ±0.4). Figure 5 shows that relative results of samples spiked with the different Cmm dilutions agree with a five-fold difference in the amount of Cmm detected by PCR (five-fold concentration difference corresponds to a theoretical ΔCq 2.3). SE-qPCR results are comparable between samples spiked with the same Cmm dilution of the series, both between replicate samples of the same seed batch as well as between samples of different batches.

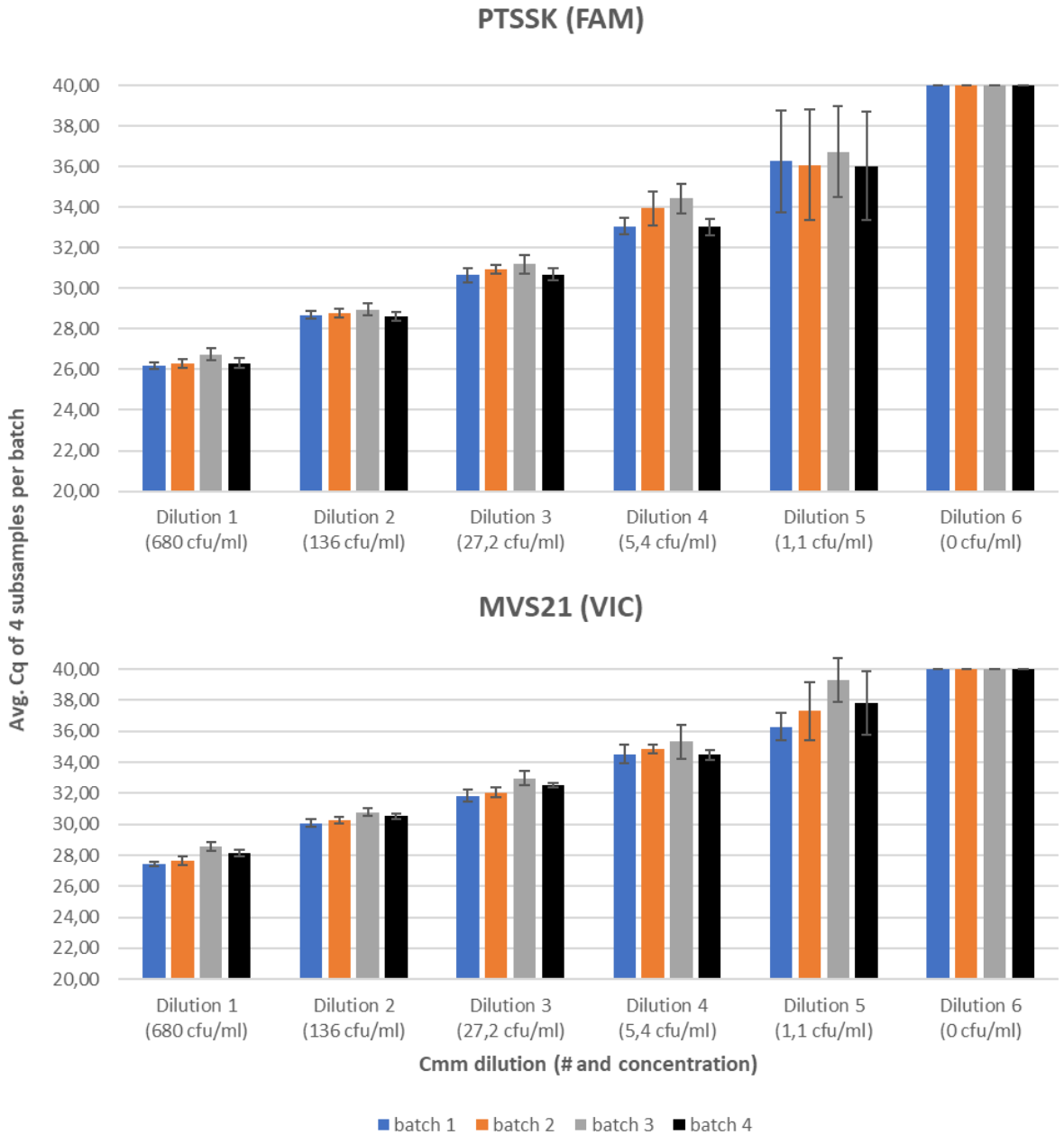

Figure 5. SE-qPCR results of test samples.
Extract prepared from four different seed batches was spiked with a five-fold dilution series of liquid cultured Cmm (dilutions 1-5). Dilution 6 did not contain any Cmm (negative control). Each Cmm dilution was spiked to four subsamples of each seed batch. The extract was processed according to the ISHI Cmm SE-qPCR protocol (Annex A). Shown are average Cq results (four subsamples per seed batch) for the two qPCR assays detecting Cmm (PTSSK and MVS21+).

All 16 samples (four batches; four subsamples) spiked with Cmm dilution 4 (5.4 CFU mL^{-1} non-concentrated seed extract) generated positive SE-qPCR results (Supplementary Table C3). Cmm dilution 5 (1.1 CFU mL^{-1} non-concentrated seed extract) was detected by SE-qPCR by both qPCR assays in 8 out of 16 samples (50%). For 14 out of 16 samples (87.5%), at least one of the two qPCR assays generated a Cq <40 (Cq 34-37; Supplementary Table C3). In the ISHI assay, a positive result is required for at least one of the two qPCR assays detecting Cmm for a SE-qPCR positive result. The LOD of the Cmm SE-qPCR assay at a 100% confidence level is 5.4 CFU mL^{-1} non-concentrated seed extract, which is comparable to that of the dilution plating assay. The SE-qPCR assay also detects 1.1 CFU mL^{-1} equally well as the plating assay, in fact detecting this Cmm-concentration in one sample more than dilution plating (respectively 14 and 13 out of 16 samples).

Conclusion

Analytical sensitivity of SE-qPCR is comparable to that of dilution plating. The LOD at a 100% confidence level is the same for both assays (5.4 CFU Cmm per mL non-concentrated seed extract). With the requirement for SE-qPCR analytical sensitivity at < 10 times the LOD of the dilution plating assay, the ISHI Cmm SE-qPCR assay is sensitive enough for its intended purpose to serve as a pre-screen for the dilution plating assay.

Results from this experiment are used to set a minimal analytical sensitivity which testing laboratories that wish to implement the ISHI Cmm SE-qPCR assay in their facilities must achieve (see Annex A, §6.1). The minimal analytical sensitivity is set at 10 CFU Cmm mL^{-1} non-concentrated seed extract that must be detected in 100% of tested samples, which meets the requirement set for analytical sensitivity of being < 10 times the LOD of the dilution plating assay. This requirement will be included in the final ISHI Cmm SE-qPCR protocol.

3.3. Selectivity

Definition ISHI guidelines: *The effect of different seed matrices on the ability of the method to detect target pathogen(s).*

The selectivity requirements will be met when a concentration of Cmm liquid cultured cells that is maximally 10 times higher than the Cmm SE-qPCR LOD is detected in samples of different seed matrices. This amount of spiked Cmm agrees with the ISHI guidelines for method validation, according to recommendations for spiking described in ISHI best practices for PCR assays (ISF-ISHI Guidelines, 2020; ISF-ISHI Best Practices, 2019). The LOD should not be significantly different between matrices. Qualitative test results should not be significantly different between seed matrices as well. Minor variability is tolerated when comparable variability is observed for detection of the PEC. Syngenta spikes Cmt strain ATCC® 33566 as a PEC, as is recommended in the ISHI Cmm SE-qPCR protocol (Annex A). SE-qPCR developmental work yielded a large collection of data for Cmt. Data indicate that Cmt detection is comparable in most seed matrices, but that detection can be lower in some matrices (data not shown).

Experimental approach

Data of the experiment described in analytical sensitivity §3.2 is used to validate SE-qPCR selectivity. Table 1 shows an overview of the experiment and §3.2 the detailed experimental approach. In short, a dilution series of Cmm liquid cultured cells was spiked to extract of four replicate samples from four different seed lots. The variation in selected batches covers requirements for validating selectivity, with differences in variety (including rootstock), country of origin and production year (see Table 4). Quantitative qPCR results of samples containing 10 – 100 times the concentration of the Cmm SE-qPCR LOD were considered to validate the selectivity criterion. Relative qPCR results for Cmm and the PEC must be comparable between samples of different seed matrices.

Results

The raw PCR results of the experiment are shown in Supplementary Table C3. Results for PCR control samples were as expected (PACs positive; NTCs non-applicable). The Cmt internal control was detected in all samples with comparable results (average Cq 29.1 ±0.4). Table 7 shows that quantitative SE-qPCR results are comparable between samples spiked with the same Cmm dilution of the series between samples of different batches (see also Figure 5 in analytical sensitivity §3.2). The Cmm SE-qPCR LOD is the same for the four seed matrices tested (100% detection of Cmm dilution 4; 5.4 CFU mL^{-1} of non-concentrated seed extract). Samples spiked with Cmm dilution 2, which contain 10–100 times the Cmm LOD concentration, show comparable relative quantitative qPCR results between Cmm and the Cmt internal control. The comparable recovery and detection of both Cmm and the Cmt internal control between seed batches confirm the absence of seed matrix effects in this experiment in detecting Cmm and Cmt by SE-qPCR.

Table 7. SE-qPCR results of test samples.
Extract prepared from four different seed batches was spiked with a five-fold dilution series of liquid cultured Cmm (dilutions 1-5 represent 680, 136, 27.2, 5.4 and 1.1 CFU ml^{-1} of seed extract, respectively). Each Cmm dilution was spiked to four replicate samples of each seed batch. The extract was processed according to the ISHI Cmm SE-qPCR protocol (Annex A). Shown are average Cq results and standard deviation (four subsamples per seed batch) for the two qPCR assays detecting Cmm (PTSSK and MVS21+). Legend: Avg: average, st dev: standard deviation, TexRed: Texas Red.

Sample (Seed batch_Cmm dilution)	Avg. PTSSK (FAM)	St dev	Avg. MVS21+ (VIC)	St dev	Avg. Cmt (TexRed)	St dev
1_1	26.19	0.17	27.45	0.15	28.93	0.21
1_2	28.70	0.19	30.08	0.26	29.11	0.21
1_3	30.64	0.35	31.84	0.38	28.44	0.28
1_4	33.05	0.41	34.53	0.58	28.52	0.17
1_5	36.26	2.51	36.30	0.90	28.41	0.15
2_1	26.28	0.22	27.63	0.28	29.21	0.20
2_2	28.78	0.21	30.25	0.19	29.61	0.32
2_3	30.95	0.20	32.06	0.33	29.27	0.34
2_4	33.94	0.82	34.85	0.29	29.20	0.27
2_5	36.09	2.72	37.30	1.88	29.18	0.21
3_1	26.73	0.30	28.53	0.28	29.22	0.24
3_2	28.96	0.29	30.77	0.24	29.46	0.24
3_3	31.20	0.46	32.96	0.44	29.07	0.24
3_4	34.43	0.73	35.34	1.10	29.56	0.24
3_5	36.74	2.23	39.30	1.40	29.16	0.14
4_1	26.30	0.24	28.15	0.23	29.19	0.26
4_2	28.61	0.23	30.52	0.18	29.38	0.23
4_3	30.69	0.30	32.50	0.14	29.17	0.08
4_4	33.02	0.39	34.48	0.30	28.93	0.06
4_5	36.04	2.67	37.83	2.06	28.99	0.10

Conclusion

Cmm is detected in all four matrices by SE-qPCR with comparable quantitative results. Samples spiked with Cmm dilution 2, which contain 10 – 100 times the Cmm LOD concentration, generate average Cq for PTSSK and MVS21+ of 28.76 (stdev 0.2) and 30.41 (stdev 0.3), respectively (Table 7). In addition, the Cmt internal control is detected in the matrices with negligible differences (all sample average Cq 29.1 stdev 0.4). No significant differences in the Cmm and Cmt detection were observed between the rootstock batch (seed batch 1) and the other three seed batches. The LOD for Cmm detection is the same for the four seed matrices tested. The ISHI Cmm SE-qPCR assay can detect both Cmm and Cmt with good selectivity.

3.4. Repeatability

Definition ISHI guidelines: *Degree of similarity in results of replicates of the same seed lots when the method is performed with minimal variations in a single lab.*

The repeatability requirements will be met when results produced for replicate samples are comparable. Accordance of test results generated must be ⩾90%.

Experimental approach

Data of the experiment described for the validation of analytical sensitivity in §3.2 was used to validate repeatability of the assay. See Table 1 for an overview of the experiment. The experiment involved spiking replicates of seed extract from four different healthy lots with liquid cultured Cmm. The objective is to validate assay elements that are specific for the SE-qPCR assay (see §2). The spiking approach is acceptable for the validation of SE-qPCR repeatability, since the procedure for extracting Cmm from seed is the same in the Cmm SE-qPCR and Cmm 4.3.1 methods. By spiking liquid cultured Cmm, the experiment is in line with ISHI guidelines of having uniformly infected replicate samples representing a range of infection levels, including one in the proximity of the assay's LOD (ISF-ISHI Guidelines, 2020). Data was generated by Syngenta, with minimal variations. Data of samples spiked with Cmm above the LOD were considered. The method described by Langton *et al.* (2002) was used to calculate the accordance of the method from qualitative data. Quantitative data for replicate samples should be within the same range.

Results

The raw PCR results of the experiment are shown in Supplementary Table C3. Results for PCR control samples were as expected (PACs positive; NTCs N/A). The Cmt internal control was detected in all samples with comparable results (average Cq 29.1 ±0.4). Table 7 shows that quantitative SE-qPCR results are comparable between samples spiked with the same Cmm dilution of the series between replicate seed samples (see also Figure 5 in analytical sensitivity §3.2). Standard deviations in results between replicate samples spiked with Cmm-concentrations above the LOD (dilutions 1-4) are small for both the PTSSK and MVS21+ qPCR assays, for all four seed matrices tested (Standard deviation ⩽1.1). The variability in the detection of the Cmt internal control between replicate samples is even smaller (Standard deviation ⩽0.34). The accordance in test results of samples spiked with Cmm dilutions 1-4 is 100%.

Conclusion

The SE-qPCR results for both Cmm and the Cmt internal control are comparable between replicate samples. The accordance of test results is 100%, showing good repeatability of the ISHI Cmm SE-qPCR assay.

3.5. Reproducibility

Definition ISHI guidelines: *Degree of similarity in results when the method is performed across labs with replicates of the same subsamples.*

The reproducibility requirements will be met when testing the SE-qPCR with replicate samples in several independent labs shows consistency between results. Concordance of test results generated must be ⩾90%, as described in ISTA guidelines (2019). In addition, the concordance odds ratio (COR) should be close to 1.

Experimental approach

A comparative test (CT) was organized between seven ISHI labs to determine the reproducibility of the SE-qPCR assay, using seed material with different Cmm infection levels (healthy, low, medium and high). See Table 2 for an overview of the experiment and the CT report in Annex B. The method described by Langton *et al.* (2002) was used to calculate the accordance, concordance, and the COR of the method from qualitative data. Testing laboratories were responsible for determining the fluorescent thresholds for their own datasets and setting a Cq cut-off value for qPCR positive results. The test organizer confirmed with participants that fluorescent thresholds were fixed just above the fluorescence background, at the start of the exponential amplification phase of PCR amplification curves. As the test organizer, Syngenta performed a homogeneity and stability test of the seed material used in the CT (Annex B).

Results

Results show homogenous and stable Cmm contamination of test material. The Cmt internal control was consistently detected by all labs in each sample. All samples were correctly scored, except for two false negative results for two out of three subsamples for a seed background with a low Cmm infection by one lab. Nevertheless, accordance and concordance of CT data generated for material with a low Cmm infection level was >90%. For the other Cmm infection levels (healthy, medium and high), accordance and concordance were 100%. See the CT report in Annex B for detailed results.

Conclusion

Results show sufficient reproducibility of the ISHI Cmm SE-qPCR assay.

3.6. Diagnostic performance

Definition ISHI guidelines: *An evaluation of the ability of the method to discriminate between positive and negative seed lots*

The diagnostic performance requirements will be met when, in reference to dilution plating, the diagnostic sensitivity of the SE-qPCR assay is 100%, demonstrating the reliability of the SE-qPCR as a pre-screen method for the dilution plating test. Compared to dilution plating, SE-qPCR diagnostic specificity must be ⩾95%.

Experimental approach

In this validation trial, samples were created that either do, or do not contain a Cmm-infected seed. Samples were tested by both SE-qPCR and dilution plating (see Table 3 for an overview of the experiment). Individual Cmm infected peduncle seeds freshly produced in 2021 were spiked to 50 subsamples of 10,000 seeds from a healthy seed lot containing a low level of saprophytes. Ten subsamples were not spiked and processed as NPCs. The SE-qPCR and dilution plating assays were performed on the same extract. The seed extract was produced according to the SE-qPCR protocol, after which 25 mL extract was sampled and processed further according to protocol (Annex A). All PCR assays were performed using the 2× PerfeCTa qPCR Toughmix (Quantabio) and CFX Opus 96 PCR machines (Bio-Rad). Data were analysed with CFX Maestro software (Bio-Rad; v2.3). For all assays, fluorescent thresholds were set at a fixed 200 RFU, and a Cq cut-off value of 35 was applied as explained in the result section. For dilution plating, according to §2-4 of ISHI Cmm method 4.3.1, non-concentrated, concentrated, and diluted seed extract was plated on two

different semi-selective media. The obtained SE-qPCR and dilution plating data were compared at the qualitative level.

Diagnostic sensitivity and diagnostic specificity of SE-qPCR were calculated in reference to dilution plating, according to the following formulas:

		Validated method result / independent assessment	
		Positive	**Negative**
Test outcome	**Positive**	True positive (TP)	False positive (FP)
	Negative	False negative (FN)	True negative (TN)
		DIAGNOSTIC SENSITIVITY = (TP / (TP + FN)) x 100%	**DIAGNOSTIC SPECIFICITY = (TN / (FP + TN)) x 100%**

Results

Raw plating results of the experiment are shown in Supplementary Table C4. All 10 NPC samples were dilution plating negative. All 50 samples spiked with a Cmm-contaminated seed were dilution plating positive. Average results of both media types show that the content of live Cmm in spiked samples ranges from 12.5-25,300 CFU mL^{-1} non-concentrated seed extract.

Raw PCR results of the experiment are shown in Supplementary Table C5. Results for PCR control samples were as expected (PACs positive; NTCs N/A). The Cmt internal control was detected in all samples with comparable results (average Cq 28.7 ±0.4). Values of Cq <40 were generated in three out of 10 NPC samples (Samples 58, 59 and 60; Cq 36.5-38.5). For two of these samples, a Cq was generated for only one of the two qPCR assays. The PCR assays were repeated for all NPC samples (data not shown). Here, Cq were again generated <40, but in three different samples. Samples 59 and 60 did not generate any Cq in the repeated PCR. Probably some NPC samples were slightly cross contaminated by the highly contaminated spiked samples during sample processing. Cross contamination in NPC is low level, and results are not reproducible between PCR assays. A Cq cut-off of 35 was applied; therefore, NPCs are validated PCR negative.

The Cmm SE-qPCR method consistently detected a single Cmm infected seed in all 50 samples of 10,000 healthy seeds, showing that diagnostic sensitivity of SE-qPCR relative to dilution plating is 100% (no false negatives). This corresponds to the requirement for a SE-qPCR assay to be a good pre-screening assay for dilution plating. The average PTSSK Cq is 22.8 ±2.0 (Cq range 19.3-26.0), the average MVS21+ Cq is 23.7 ±2.0 (Cq range 20.5-26.7). In addition, all 10 NPC samples were negative in both assays, showing diagnostic specificity of the SE-qPCR assay to be 100%.

Conclusion

All 50 samples spiked with a Cmm contaminated seed were both dilution plating- and SE-qPCR positive, while all 10 NPC samples were negative for both assays. The SE-qPCR analytical sensitivity is such that it detects a single Cmm contaminated seed spiked to a sample of 10,000 healthy seeds. In reference to dilution plating, SE-qPCR diagnostic sensitivity and specificity are 100%. The diagnostic performance of SE-qPCR is good, confirming it to be a suitable pre-screening assay for dilution plating.

4. CONCLUSION

The data presented in this validation report show that the SE-qPCR detection assay for Cmm in samples of tomato seed meets all requirements set for the validation criteria analytical specificity, analytical sensitivity, selectivity, repeatability, reproducibility, and diagnostic performance. It is concluded that the ISHI Cmm SE-qPCR assay is fit for its intended purpose to serve as an optional pre-screening assay for the ISHI Cmm dilution plating assay.

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6. ANNEXES

Annex A: Cmm SE-qPCR protocol

Introduction

This SE-qPCR pre-screening method was initially developed and validated by Naktuinbouw in the framework of the EU TESTA project https://cordis.europa.eu/project/rcn/105068/reporting/en). DNA purification using a widely available kit replaces automatic magnetic bead purification in the Naktuinbouw protocol.

Recommended sample size (10,000 seeds), maximum subsample size (10,000 seeds as well) and sample preparation are according to the ISHI method for the detection of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) on tomato seed containing the dilution plating assay (ISHI Method Cmm 4.3.1, 2017). Hence, if desired, a single seed sample can be processed by both the SE-qPCR and dilution plating assays. Samples should however be processed at the same time by both methods, since the effect of storing seed extract on test results is not fully known.

This SE-qPCR pre-screening method is meant to be incorporated in the overall ISHI method for Cmm, together with the follow-up dilution plating assay.

As the format of ISHI protocol descriptions is subject to change, the description below is limited to the SE-qPCR pre-screening. It is the method selected to be further validated in ISHI and descriptions may be more specific than in the final ISHI protocol. Controls not specified will be incorporated in the comparative test as per ISHI best practices for PCRs in seed health tests.

Detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato by a seed extract PCR (SE-qPCR) pre-screening assay

Materials

- Stomacher machine and bags
- Seed extraction buffer (Table A.1)
- Phosphate Buffer (Table A.2)
- Refrigerator at 4 °C
- Centrifuges capable of spinning 15/50 mL conical tubes and 1.5/2 mL microtubes (Optional: microtiter plates), at the required RCF
- *Clavibacter michiganensis* subsp. *tessellarius* (Cmt) ATCC® 33566
- Tissuelyser II or Genogrinder
- Dry Low binding Ø 0,1 mm zirconium/glass beads (SPEX SamplePrep)
- DNeasy® Blood & Tissue single column kit (Qiagen)
- Heating block for 1.5/2 mL microtubes (shacking, otherwise additional vortex is required)
- qPCR mix, primers (Table A.3) and equipment
- Lab disposables

Table A.1. Seed extraction buffer[a] (pH 7.4)

Compound	Amount/L
Na_2HPO_4	7.75 g
KH_2PO_4	1.65 g
Tween 20	0.20 mL
$Na_2S_2O_3$[b]	0.50 g

[a]Use de-ionized water, and autoclave at 121 °C, 15 psi for 15 min

[b]Recommended when seeds have been treated with hypochlorite

Table A.2. Phosphate buffer[a] (PB) (pH 7.4)

Compound	Amount/L
Na_2HPO_4	7.75 g
KH_2PO_4	1.65 g

[a] Use de-ionized water, and autoclave at 121 °C, 15 psi for 15 min.

Table A.3. Primer-sequences and references

Name	Sequence	Source
MVS21-F	5' - CTA GTT GCT GAA TCC ACC CAG - 3'	Sudarshana *et al.* 2012
MVS21+-R*	5' – GTA CCG CTT GAC TCT CGT TTC - 3'	
MVS21+-Pr**	5' - VIC - CTG CCA CCC GAT GTT GTT GTT CCG - BHQ1- 3'	
PTSSK-F	5' - CGT CGC CCG CCC GCT G - 3'	Berendsen *et al.* 2011
PTSSK-R	5' - GGG GCC GAA GGT GCT GGT G - 3'	
PTSSK-Pr	5' - FAM - TGG TCG TCC TCG GCG - MGB - NFQ - 3'	
Cmt-F	5' - AAC CCC AGG TCG TCT TGT CGA A - 3′	Naktuinbouw 2016*a*
Cmt-R	5' – GCG CGT CTA CAC GGG CAT CA - 3′	
Cmt-Pr	5' – TexRed - TGT CGT CGA TCC AGG CCT CGC CC - BHQ2 - 3'	

* Original MVS21 reverse primer extended with single G-nucleotide at 5'-end

** Original MVS21 reverse probe extended with single G-nucleotide at 3'-end

1. Extraction of bacteria from the seed

1.1. Put seed subsamples in sterile stomacher bags with filter (e.g., Grade Separator 400 blender bags (UK)). Add sterile seed extraction buffer to each bag at a ratio of 4 mL of seed extract buffer per gram of seed (v/w).

1.2. Incubate overnight (minimum 14 hours) at 4 °C, and macerate for at least 4 min in a stomacher machine until the extraction buffer becomes milky and white particles become visible as a result of endosperm release (see Figure A.1).

Figure A.1. The seed extraction buffer and seeds after stomaching

2. Collection of target bacteria by differential centrifugation

2.1. From behind the filter, transfer 2.5 mL of extract per 1,000 seeds in the subsample to a suitable centrifuge tube.

Note: if plating is desired to be done on the same extract, more of this extract can be processed for this purpose. Process extract intended for testing by dilution plating according to the ISHI Method Cmm v4.3.1. Do not spike with the PEC bacterium, for it will interfere the manifestation and recognition of Cmm colonies on medium plates. Start processing extract by dilution plating within 30 min after centrifugation.

2.2. Spike each subsample extract with 10,000 cells of *Clavibacter michiganensis* subsp. *tessellarius* (Cmt) ATCC® 33566. The bacterial suspension for spiking may be prepared fresh or in advance (stored at -80 °C in a 15% (v/v) glycerol solution).

2.3. Further clear the extract from debris by low-speed centrifugation at 1,000 × *g* for 1 min. This facilitates resuspension of the pellets obtained in step 2.6.

2.4. Decant the supernatant into a new centrifugation tube and discard the pellet.

2.5. Centrifuge the supernatant with a time and speed sufficient to pellet bacteria (e.g., at 5,000 × *g* for 15 min).

2.6. Remove the supernatant carefully and resuspend the pellet in 1-1.5 mL phosphate buffer (PB, Table A.2).

3. Cell lysis and DNA isolation

3.1. Transfer the cell suspension obtained in step 2.6 to 2 mL Safelock tubes with round bottom. Spin down (e.g., at 5,000 × *g* for 5 min) the cells and discard the supernatant. Resuspend the pellet in 230 µL phosphate buffer (PB). Add 360 µL of dry Ø 0,1 mm zirconium/glass beads (BioSpec Products, Inc.) and beat in a Qiagen Tissuelyser II at 30 Hz for 3 min or in a Genogrinder at 1,500 rpm for 3 min. Short spin the samples to collect beads and liquid at the bottom of the tubes.

Note: DNA isolation is done using the DNeasy® Blood & Tissue kit (Qiagen), but with modifications. The below instructions are tailored for the DNeasy Mini Spin Columns. The

quality of DNA samples prepared with the DNeasy Blood & Tissue 96 well plate kit was lower (Syngenta data, not shown); therefore, it is not recommended to use the 96 well plate format of the kit with this method. Spin columns and plate were used according to instructions in the DNeasy® Blood & Tissue Handbook (Qiagen). Protocol-specific instructions follow below.

Note: Modifications are the consequence of bacterial lysis by bead beating being specific for this protocol and which is done in a detergent-free buffer to limit foam generation.

Note: zirconium/glass beads bind DNA, reducing yield with low input seed extract amounts. Low binding Ø 0,1 mm zirconium beads (SPEX SamplePrep) could be used as an alternative when recovery of the spiked positive extraction control (PEC) is low.

3.2. Add 205 µL buffer ATL and 25 µL proteinase K to each tube and incubate at 56 ˚C (minimum 3 hours to maximum overnight (~16 hours)). Vortex at least every hour or use a shaking incubator. Do not premix buffer ATL and proteinase K more than 10-15 min before addition to the sample. Proteinase K tends to self-digest in buffers without substrate. Short spin the samples to collect beads and liquid at the bottom of the tubes

3.3. Add 460 µL buffer AL and mix by vortexing. Short spin samples to collect beads and liquid at the bottom of the tubes. Pipet 560 µL lysate without taking any beads and mix with 280 µL EtOH [96-100% (v/v)] in a clean reaction tube and mix by vortexing.

3.4. Continue with procedure step 4 of the protocol "Purification of Total DNA from Animal Tissues (Spin-Column Protocol)" in the DNeasy® Blood & Tissue Handbook. Load and spin the 840 µL volume in two portions, not exceeding 600 µL for either portion. Empty the collection tube after the first portion to accommodate the flow-through of the second. Do this with care to minimize risk of cross-contamination.

3.5. Finally, elute DNA in 100 µL buffer AE.

4. qPCR assay

4.1. Perform, in duplicate, a multiplex qPCR reaction on DNA of each subsample, combining the two Cmm qPCR assays with the Cmt qPCR assay, which serves as the IAC as well as for detection of the PEC. Primer and probe compositions are shown in Table A.3.

4.2. Add 5 µL template on a total reaction volume of 25 µL, with a concentration of 0.40 µM of primers and probes, except for the Cmt probe which should have a concentration of 0.20 µM.

4.3. PCR conditions: 10 min incubation at 95 °C followed by 40 cycles of 15 sec at 95 °C and 48 sec at an annealing temperature of or around 67 °C. Ramp speed at 5 °C/s.

Note: the annealing temperature is critical and should be optimized for the PCR mix used. Since the MVS21+ qPCR primers have a lower Tm than those of the PTSSK qPCR an annealing temperature gradient should be performed to select the temperature 1 °C lower than the point where MVS21+ qPCR Cq values start to increase.

5. qPCR assay evaluation

5.1. A sample is considered suspect for Cmm if one or more subsamples show detection of Cmm DNA by one or both Cmm primer sets. It is the responsibility of the testing laboratory to determine threshold/cut-off values, such that a minimal analytical sensitivity is achieved.

Testing laboratories need to achieve 100% detection of Cmm in a concentration of 10 CFU/mL non-concentrated seed extract by one or both Cmm primer sets.

5.2. If a sample is determined qPCR Cmm suspect, and Cmm dilution plating was not performed on the same extract in parallel, the Cmm dilution plating method must be performed on a new seed sample to reach a conclusion about the sample and seed lot (see ISF view on indirect seed health tests.)

Annex B: Comparative test report

1. Organization and timeline

1.1. Test organization

Jeroen Lastdrager (Syngenta)

1.2. Participating labs:

Laboratory	Contact	Country	Remarks
Syngenta	Jeroen Lastdrager	Netherlands	Organizer
Hazera	Hila Danino	Israel	
Takii	Shintaro Kusano	Japan	
Anove	Jerson Garita	Spain	
GEVES	Thomas Baldwin	France	
Bayer	Jill Demers	USA	
ENZA	Debby Beugelsdijk	Netherlands	
Bayer	Louis Sorieul	Netherlands	

Criteria for participation

Participating laboratories were required to be proficient in seed health testing and show experience in performing the Cmm SE-qPCR protocol (data not shown).

2. Background and objectives

Clavibacter michiganensis subsp. *michiganensis* (Cmm) causes wilting in tomato (*Lycopersicum esculentum*). Viable Cmm is currently being detected in seed samples by stomaching seeds in buffer and plating dilutions of resulting seed extract on semi-selective solid growth medium. Suspect colonies of Cmm are confirmed by real-time PCR (qPCR) (ISHI protocol for detection of *Clavibacter michiganensis* subsp. *michiganensis* on tomato seed).

Naktuinbouw developed and validated a seed extract PCR (SE-qPCR) protocol, in which seed extract is prepared in the same way as for the dilution plating section of Cmm method 4.3.1. Bacteria are collected and subjected to a cell lysis step, after which DNA is isolated and analysed by real-time qPCR. The Naktuinbouw protocol was used as the basis for the development of an ISHI standard protocol for detection of Cmm by SE-qPCR (Annex A).

The objective of this comparative test (CT) was to validate the reproducibility of the ISHI Cmm SE-qPCR assay in detection of Cmm DNA extracted from samples of untreated or acid treated tomato seeds by qPCR. The aim of validation was to deliver an ISHI internally accepted standard pre-screening protocol for testing tomato seeds for the presence of *Clavibacter michiganensis* subsp. *michiganensis*. A negative result in the pre-screen is final, and a positive result is followed up by performing dilution plating to confirm the presence of viable bacteria.

3. Test design

The CT was designed according to the ISHI method validation guidelines (ISF-ISHI guidelines, 2020). The CT was performed independently by members of the seven participating labs. Identity of test material was only known to the test organizer. Seed material consisted of six different backgrounds. Backgrounds include healthy seed material as well as material with different levels of Cmm infection (high-medium-low; Table B1). Cmm infection levels were obtained by mixing

material of infected seed lots with healthy seeds. All seed material was treated with hydrochloric acid. Categorization of infection levels was based on experimental data obtained with infected seed lots (Syngenta report, 2020*d*). Each lab tested three replicate samples from each seed background, so 18 test samples in total.

Table B1. Composition of tomato seed backgrounds provided to each participant of the CT.
Total number of test samples per participant was 18 (three subsamples from six different seed backgrounds). Seed backgrounds were obtained by mixing seed from a healthy seed lot (lot# 12954469) with seed from naturally infected seed lots (lot# 10374103 (PTSSK and MVS21 Cq 29) and 10428654 (PTSSK and MVS21 Cq 31), see Syngenta report 2020*d*). The resulting seed mixtures ranged in the level of Cmm infection. Seed background A was healthy, free from Cmm infection (N/A at 40 PCR cycles), seed backgrounds B and C were low infected, seed backgrounds D and E were medium infected, and seed background F was highly infected.

Seed background	Cmm infection level	Sub samples	Sample codes	Composition
A.	Healthy (NPC)	3	8-12-13	10,000 seeds batch 12954469
B.	Low	3	4-7-17	9,500 seeds batch 12954469 + 500 seeds batch 10374103
C.	Low	3	1-2-14	9,500 seeds batch 12954469 + 500 seeds batch 10428654
D	Medium	3	10-11-16	9,000 seeds batch12954469 + 1,000 seeds batch 10374103
E.	Medium	3	3-6-18	10,000 seeds batch 10428654
F.	High	3	5-9-15	10,000 seeds batch 10374103
	Total #samples	**18**		

Samples of 10,000 seeds were prepared by the CT organizer. Prior to sending, the test organizer performed homogeneity testing of the material by processing 10 subsamples of 10,000 seeds from each of the six seed backgrounds by SE-qPCR (Annex A). Seedcalc8 software was used to calculate the infection rate of each seed lot and the probability for having positive test results. Afterwards, the CT material was distributed to participating labs and advised for storage under controlled conditions upon arrival (4-15 °C). Table B2 summarizes the schedule for sending the CT test material, days of travel to each participant and the date of CT finalization. Participating labs were randomly numbered. Numbering is consistent in the results section of this report.

Table B2. Schedule of CT test material shipment to participating labs and CT test performance. Participants were randomly numbered. Test organizing lab (Syngenta) is excluded in this overview.

Lab	Shipment (date)	Days of travel	Test finalization (date)	Days between shipment and test finalization
1.	23-11-2021	2	24-1-2022	62
2.	26-01-2022	1	10-2-2022	15
3.	23-11-2021	24	22-12-2021	29
4.	30-11-2021	1	27-1-2022	58
5.	23-11-2021	2	21-1-2022	59
6.	23-11-2021	7	19-1-2022	57
7.	23-11-2021	14	18-1-2022	56

Samples of the healthy seed background double functioned as Negative Process Control (NPCs), and samples of the highly infected background also served as Positive Processing Controls (PPCs). Participants used their own key equipment and consumables. Reference material for the PEC/IAC and for PCR positive amplification control (PAC) DNA were also provided by the participating lab. According to the ISHI Best practices for PCR testing, the use of a PEC/IAC and PACs is essential, as well as a Non-Template Control (NTC) (ISFI-ISHI Best practices, 2018). All CT participants used Cmt as PEC/IAC. A template Excel sheet was provided to participants in which an overview of all replicates of test samples and controls was given, and in which CT data was collected. Samples were randomly coded. Sample identity was only known by the test organizer. After the CT was completed by all participants, test organizer performed stability testing of the material by again processing 10 subsamples of 10,000 seeds from each of the six seed backgrounds by SE-qPCR (Annex A). Results of the stability test were compared to the expected number of positive results as calculated in the homogeneity test, determining the stability of the infection status of samples between sample packaging and finalization of the CT.

The ISHI protocol for Cmm SE-qPCR, as shown in Annex A, was followed by all participants. All results generated were shared with the test organizer and collected in the provided template Excel datasheet. Raw RT-qPCR quantitative data as well as qualitative results for each sample (Cmm positive or negative) were reported by each lab. Participants were asked to determine the Cq cut-off value for qualitative test results for their own dataset. Qualitative test results were used to determine both repeatability and reproducibility of the ISHI Cmm SE-qPCR assay. Accordance (repeatability of replicate sample results within each lab) and concordance (reproducibility of results between labs) of CT data were calculated for each Cmm infection level, using the method published by Langton *et al.* (2002).

4. Results

4.1. Homogeneity and stability tests

For each of the six seed backgrounds used in the CT, SE-qPCR data for 10 subsamples was generated by the CT organizer (Syngenta) according to the ISHI Cmm SE-qPCR assay (Annex A). PCR assays were performed using the 2× PerfeCTa qPCR Toughmix (Quantabio) and CFX Opus 96 PCR machines (Bio-Rad). Data was analysed with CFX Maestro software (Bio-Rad; v2.3). For all assays, fluorescent thresholds were set at a fixed 200 RFU, and a Cq cut-off value of 35 was applied.

Raw homogeneity and stability data are shown in Supplementary tables C6 and C7. Results for PCR control samples were as expected (PACs positive; NTCs N/A). Table B3 shows the average quantitative results of the 10 subsamples per seed lot and qualitative results of both tests. The Cmt internal control was detected in all samples with comparable results in both homogeneity and stability tests. Qualitative test results are the same in both tests for all six seed backgrounds (subsamples of healthy background negative, all subsamples of the other five backgrounds positive). All three levels of Cmm contamination were detected consistently, showing homogenous Cmm contamination of the test material. The Cq values generated in homogeneity and stability tests are comparable as well, showing contamination was stable during the course of the CT. Except for seed background D in the homogeneity test, variability between subsamples is very low (Standard deviation ⩽0.5). One of 10 subsamples of seed background D was substantially more positive compared to the others (PTSSK and MVS21+ Cq 23, see Supplementary Table C6).

Table B3. Quantitative and qualitative homogeneity- and stability test results of CT material.
Prior to supplying participants- and after the CT was completed by all participants, 10 subsamples of 10,000 seeds from each of the six seed backgrounds included in the CT were processed by SE-qPCR (Annex A). For seed sample composition, see Table B1. Shown are average Cq results (±Standard deviation) for the two qPCR assays detecting Cmm (PTSSK and MVS21+) and the assay detecting the Cmt internal control. For each seed background tested, the number of subsamples positive for Cmm are indicated.
Legend: Avg.: average, pos: positive, subs: subsample

Seed lot	Cmm infection level	Homogeneity				Stability			
		Avg. PTSSK (FAM)	Avg. MVS21+ (VIC)	Avg. Cmt (TxRd)	Pos. subs of total	Avg. PTSSK (FAM)	Avg. MVS21+ (VIC)	Avg. Cmt (TxRd)	Pos. subs of total
A.	Healthy (NPC)	40.0 (±0)	40.0 (±0)	28.8 (±0.3)	0/10	40.0 (±0)	40.0 (±0)	29.6 (±0.3)	0/10
B.	Low	30.2 (±0.3)	29.6 (±0.2)	28.7 (±0.3)	10/10	31.1 (±0.2)	30.3 (±0.2)	29.0 (±0.3)	10/10
C.	Low	30.6 (±0.3)	31.3 (±0.5)	28.9 (±0.3)	10/10	31.5 (±0.4)	31.7 (±0.5)	29.1 (±0.5)	10/10
D	Medium	28.5 (±1.9)	28.1 (±1.9)	28.5 (±0.3)	10/10	29.7 (±0.2)	29.3 (±0.3)	29.4 (±0.4)	10/10
E.	Medium	27.4 (±0.3)	28.2 (±0.2)	29.0 (±0.4)	10/10	27.9 (±0.3)	28.4 (±0.3)	29.4 (±0.4)	10/10
F.	High	25.2 (±0.5)	25.1 (±0.5)	29.0 (±0.3)	10/10	26.0 (±0.4)	25.5 (±0.4)	29.8 (±0.5)	10/10

For the homogeneity test it is concluded that the infection rate of each Cmm-contaminated seed background included in the CT (backgrounds B-F) is 100%. All subsamples of these backgrounds are expected to be positive in the CT. For the healthy seed background A, no subsamples are expected to be positive. Based on the stability test, it is concluded that the infection status of all seed backgrounds is stable between sample packaging and finalization of the CT.

4.2. Comparative test

Each CT participating lab tested three replicate samples from six different seed lots: 18 test samples in total. Reference material for the PEC/IAC and for PCR positive amplification control (PAC) DNA were provided by the participating lab, and participants determined the Cq cut-off

value for qualitative test results for their own dataset (Table B4). CT organizer confirmed with participants that fluorescent thresholds were set for each qPCR assay in such a way that they intersect qPCR amplification curves in the exponential phase (data not shown). Raw CT data are shown in Supplementary Table C8.

Table B4. Cq cut-off values applied by each CT participating lab.

Lab	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)
1.	⩽35	⩽35	⩽35
2.	⩽35	⩽35	⩽35
3.	<37	<37	<37
4.	<35	<35	<32
5.	<36	<36	<30
6.	<35	<35	<35
7.	<40	<40	<40

Results of control samples are shown in Table B5. NTCs were negative for all three qPCR assays for each lab. Except lab 4, all labs used DNA extracted from cultured isolates as PACs. Lab 4 did do this for the PAC for the Cmt IAC, but used DNA isolated from a negative seed background spiked with both Cmm and Cmt cells as Cmm-PAC. This explains why lab 4 is the only lab with a Cmt qPCR assay positive result for the Cmm PAC (Cq 27.8). All labs generated PTSSK and MVS21+ qPCR assay positive results for the Cmm PAC. For all labs except lab 5, PTSSK and MVS21+ assays were negative and the Cmt qPCR assays were positive for the Cmt PAC. Lab 5 did not include a Cmt PAC in the PCR analysis, but instead concluded that the PCR analysis was successful based on Cmt qPCR results of spiked test samples (Table B6 and Supplementary Table C8). Labs were responsible for providing their own PAC samples, which explains the considerable variability in PAC quantitative qPCR results between labs. Nonetheless, PACs of all labs generated positive qPCR results and therefore performed according to the intended purpose. Control results confirm that the qPCR data provided by CT participants is of sufficient quality to validate the reproducibility of the ISHI Cmm SE-qPCR assay.

Table B5. CT test results for control samples.
Control samples were provided by testing laboratories themselves and not by the test organizer. Positive amplification control samples (PACs) for Cmm and Cmt and Negative Template Control samples (NTCs) were included in the PCR analysis by each lab, except by lab 5, which did not include a PAC for the Cmt internal control. The Cq results for the two qPCR assays detecting Cmm (PTSSK and MVS21+) and the assay detecting the Cmt internal control for the PACs are shown here. The NTC data is not included in the table: results were N/A for all labs. Cq values represent the average of duplicate PCR reactions ±Standard deviation. Except for lab 7, PCR programs included 40 cycles. Lab 7 used 45 cycles.
* Lab 4 used DNA isolated from samples of a negative seed background spiked with Cmm and Cmt cells as Cmm-PAC.

Lab	PAC Cmm			PAC Cmt		
	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)
1.	20.9 (±0.0)	22.3 (±0.0)	40.0 (±0.0)	40.0 (±0.0)	40.0 (±0.0)	18.9 (±0.1)
2.	26.8 (±0.4)	27.6 (±0.0)	38.4 (±0.0)	40.0 (±0.0)	40.0 (±0.0)	29.6 (±0.1)
3.	28.1 (±0.3)	29.2 (±0.1)	40.0 (±0.0)	40.0 (±0.0)	40.0 (±0.0)	27.3 (±0.0)
4.	*30.5 (±0.0)	*29.6 ±0.1)	*27.8 (±0.1)	40.0 (±0.0)	40.0 (±0.0)	28.9 (±2.3)
5.	18.1 (±0.2)	18.3 (±0.5)	40.0 (±0.0)	-	-	-
6.	20.8 (±0.3)	19.0 (±0.2)	40.0 (±0.0)	40.0 (±0.0)	40.0 (±0.0)	30.8 (±0.4)
7.	23.0 (±0.0)	23.4 (±0.0)	45.0 (±0.0)	45.0 (±0.0)	45.0 (±0.0)	27.3 (±0.0)

Labs performed PCR reactions in duplicate. With few exceptions, PCR duplicate results for CT test samples are comparable for each CT participating lab (Supplementary Table C8). All labs successfully detected the Cmt internal control in all test samples (Figure B1). Outliers in the figure for labs 2 and 6 represent the result for one of the two PCR duplicates of a sample. Overall, labs generated comparable Cmt results between test samples of different matrices. This shows that Cmt is detected with good selectivity, without noticeable seed matrix effects. Cmt detection by labs 5 and 6 was more variable. Between labs 2, 3, 4 and 7, Cmt results are comparable, indicating similar recovery and detection of the Cmt spike. Labs 1 and 5 generated lower Cq values for Cmt. The SE-qPCR protocol prescribes to spike 10,000 cells of Cmt to each sample: possibly labs 1 and 5 spiked more. Conversely, it is possible that lab 6 spiked with fewer cells, resulting in relatively high Cmt Cq values. Alternatively, Cmt recovery and detection by lab 6 was, in general, lower relative to other labs. Nevertheless, detection of the Cmt internal control by all labs was sufficient. In conclusion, based on the Cmt internal control results, all test results are valid and can be used to conclude on the reproducibility of the SE-qPCR assay.

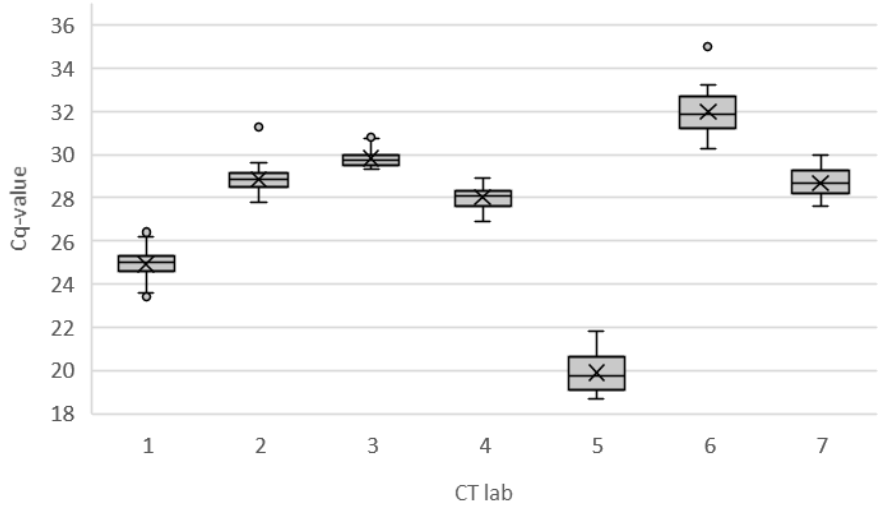

Figure B1. Box and whisker plots of Cmt internal control SE-qPCR results for CT test samples.
For each CT lab, data represents Cmt qPCR results for all test samples combined (three subsamples of six seed backgrounds analysed by qPCR in technical duplicates). X-markers indicate data means (*Lab 1* Cq 24.9; *Lab 2* Cq 28.9; *Lab 3* Cq 29.8; *Lab 4* Cq 28.0; *Lab 5* Cq 19.9; *Lab 6* Cq 32.0; *Lab 7* Cq 28.7). Circles indicate outliers.

Cq values generated by CT participants are generally higher compared to the homogeneity results generated by the test organizer (Figure B2, Table B3). However, like for the homogeneity data, the quantitative PCR results shown in Figure B2 do reflect the differences in Cmm infection levels between seed backgrounds. Labs 2, 3 and 6 generated Cq results above the Cmm cut-off value for healthy seed background A, as shown by the outliers in PTSSK and MVS21+ data for sample A in Figure B2. After discussion with CT participants, it was concluded that these outliers are likely the result of cross contamination from Cmm positive samples processed in the CT. The background A outlier of Cq 45 is explained by the fact that lab 7 ran a PCR program of 45 cycles, as all other participants ran 40 cycles.

Overall, the PTSSK dataset includes more outliers compared to that of MVS21+. Variability in PTSSK PCR results between labs is largest for seed backgrounds C (low Cmm) and E (medium Cmm). Variability is unlikely to be caused by differences in infection level between subsamples, as the data in Table B3 shows that the material is homogeneously infected. As is shown by Supplementary Figure C1, labs 5 and 7 generate higher Cq values for test samples compared to the other participating labs, largely explaining the variability in qPCR results. The higher Cq values generated by labs 5 and 7 probably explain why these labs, together with lab 3, applied higher Cmm cut-off values than other labs (>36; Table B4). Lab 7 applied a Cq cut-off <40, explaining why this lab correctly scored all subsamples of seed background C Cmm positive, although lab 7 generates higher Cq values for background C samples compared to lab 5.

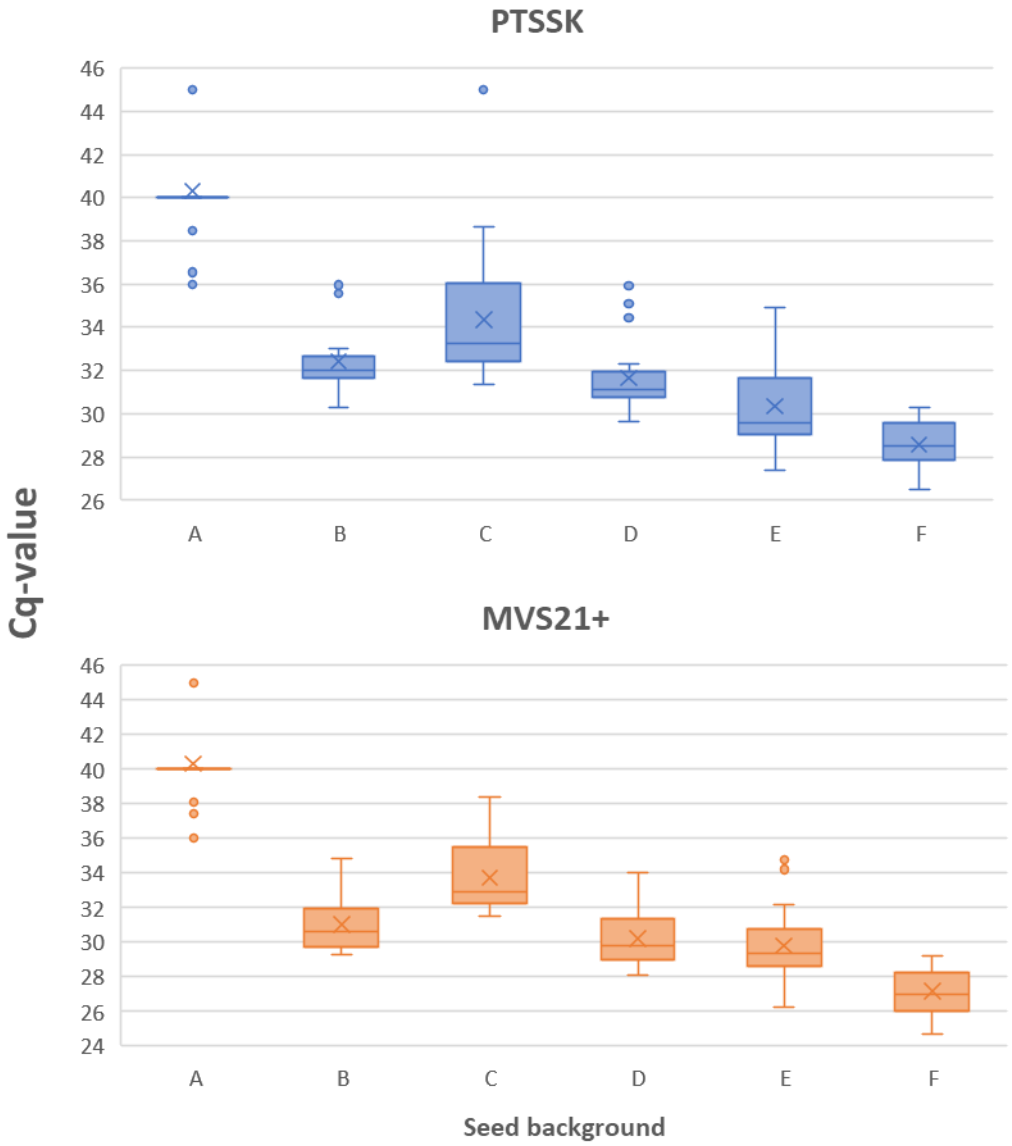

Figure B2. Box and whisker plots of PTSSK and MVS21+ SE-qPCR results for CT test samples. Seed background A is a healthy batch, B-F are Cmm infected. Backgrounds B and C are low level infected; D and E are medium level infected, and background F is highly infected. For each seed background, data represents qPCR results from all test samples of each lab combined (seven labs analysed three subsamples in PCR technical duplicates). Legend: X-markers indicate data means. Circles indicate outliers.

Table B6 summarizes the qualitative CT results. Based on the Cq cut-off value for positive PCR results set by participating labs themselves, all labs were able to correctly identify all subsamples of seed background A as healthy. All subsamples of seed backgrounds B, D, E and F were correctly identified as Cmm positive. Apart from lab 5, all participants scored all subsamples of seed background C Cmm positive as well.

Table B6. Qualitative results for CT test samples per participating laboratory (1-7) and seed background (A-F).
Seed backgrounds varied in Cmm-infection; the infection level is indicated. Results represent the number of subsamples found positive for Cmm relative to the total number of subsamples tested. Expected qualitative results are given. Laboratories set the Cmm Cq cut-off value for positive PCR results themselves (see Table B4).

	Seed lot A. (Healthy)	**Seed lot B. (Low)**	**Seed lot C (Low)**	**Seed lot D (Medium)**	**Seed lot E (Medium)**	**Seed lot F (High)**
Expected result	0/3	3/3	3/3	3/3	3/3	3/3
Lab 1.	0/3	3/3	3/3	3/3	3/3	3/3
Lab 2.	0/3	3/3	3/3	3/3	3/3	3/3
Lab 3.	0/3	3/3	3/3	3/3	3/3	3/3
Lab 4.	0/3	3/3	3/3	3/3	3/3	3/3
Lab 5.	0/3	3/3	1/3	3/3	3/3	3/3
Lab 6.	0/3	3/3	3/3	3/3	3/3	3/3
Lab 7.	0/3	3/3	3/3	3/3	3/3	3/3

Lab 5 generates overall higher Cq values for the test material as compared to other participating labs (Supplementary Figure C1). Possibly, the recovery of Cmm from CT test samples was generally lower. Lab 5 used a Cmm Cq cut-off of 36 and scored one out of three subsamples of seed background C Cmm positive (sample code #1; PTSSK Cq 36,1 and 36.9 and MVS21+ Cq 33.6 and 35.42 for PCR duplicates). Results for the other two subsamples of seed background C generated Cq values >36 (sample code #2; PTSSK Cq 38,7 for both PCR duplicates, and MVS21+ Cq 36.4 and 36.01 for PCR duplicates. sample code #14; PTSSK Cq 36,9 and 36.03 and MVS21+ Cq 37.2 and 36.03 for PCR duplicates). See also Supplementary Table C8.

Of the two subsamples of seed background C that were wrongfully scored as Cmm negative by lab 5, MVS21+ qPCR results for one of the two PCR duplicates were only <0.1 Cq over the Cq cut-off of 36. Setting the cut-off at Cq 37 instead would result in Cmm positive results for both PCR duplicates of subsamples #1, 2 and 14 of background C. In comparison, lab 5 generated all negative PCR results for subsamples of healthy background A (Cq ⩾40). The fact that quantitative PTSSK and MVS21+ qPCR results of lab 5 for seed background A (healthy) are negative, while those of background C (low Cmm infection) are positive, would support a similar Cmm validation at a qualitative level. However, Cq threshold setting was part of this CT, and is part of the ISHI SE-qPCR assay as well (Annex A). Therefore, the repeatability and reproducibility of CT data are validated using the original qualitative results reported by the CT participants (Table B6).

The accordance (repeatability), concordance (reproducibility) and concordance odds ratio (COR) was calculated for each Cmm infection level, using the method published by Langton *et al.* (2002). Results are shown in Table B7. Accordance and concordance of CT data for healthy, medium and high Cmm infection levels is 100%, with a COR of 1.0. Low Cmm infection level data accordance and concordance are > 90%, with a COR of 1.28. Inter- and intra-laboratory results are consistent and meet the requirements set for SE-qPCR reproducibility.

Table B7. Accordance, concordance and concordance odds ratio (COR) for CT material per Cmm infection level, calculated according to Langton *et al.* (2002).

Cmm infection level	Seed background(s)	Accordance	Concordance	COR
Healthy	A	100%	100%	1.0
Low	B and C	92.4%	90.5%	1.28
Medium	D and E	100%	100%	1.0
High	F	100%	100%	1.0

5. Conclusions

As was expected based on homogeneity testing, all CT labs successfully scored subsamples of healthy background A as Cmm negative. The high Cq values generated for some subsamples of background A by labs 2, 3 and 6 were above the Cmm cut-off value and were therefore scored negative.

Homogeneity and stability testing results lead to the expectation that all subsamples of all Cmm-infected seed backgrounds would be positive in the CT (high, medium and low Cmm infection level). Cmm qPCR Cq values generated by CT participants are higher compared to the homogeneity results generated by the test organizer. However, like the homogeneity data, the quantitative PCR results reflect the differences in Cmm infection levels between backgrounds. All labs successfully identified all subsamples of Cmm infected backgrounds B-F as Cmm positive, except for lab 5. Two subsamples of Cmm low infected seed background C were incorrectly scored as Cmm negative by this lab. It is unlikely lab 5 received subsamples of seed background C that had a lower Cmm infection than other labs. Fluorescent threshold setting was not a contributing factor since each lab showed that thresholds for each qPCR assay were set in such a way that they intersect qPCR amplification curves in the early exponential phase (data not shown).

Lab 5's Cq values for the two incorrectly scored subsamples of background C were just over the Cmm cut-off value of 36. No PCR signals were generated for truly negative subsamples (seed background A), so it could be argued that lab 5 did find all subsamples of seed background C Cmm positive when applying a slightly higher cut-off value, while this would not lead to any false positive results for samples of seed background A. However, Cq cut-off setting is part of the performing lab's responsibility (Annex A); therefore, the two false negative results for seed batch C were maintained when determining CT data's repeatability and reproducibility. Accordance and concordance were both >90% for each Cmm infection level used in the CT, showing good repeatability and reproducibility of the ISHI Cmm SE-qPCR assay.

Annex C: Supplementary Tables C1-C8 and Figure C1

Table C1. Plating results of the Cmm five-fold dilution series on non-selective agar medium plates (analytical sensitivity §3.2).
The series consisted of five Cmm-containing dilutions and one free from Cmm (Dilution 6; negative control; 0 CFU). The Cmm concentration of the series was determined in two ways: 1). Dilutions 4, 5 and 6 were directly plated in triplicate on non-selective agar medium (100 µL/plate), and 2). Dilutions 1-5 were diluted in Phosphate Buffer (PB) (see Table A2 for recipe) in the same ratio as they were spiked to test samples in the experiment (1.4 µL spike/mL). Plates were incubated at 27 °C and scored after four days. Results of replicate plates were averaged. The Cmm concentration of the series (CFU/1.4 µL) was calculated based on the colony-count of the dilution 1; diluted further in buffer. Concentrations correspond to the amount of Cmm spiked per mL non-concentrated seed extract.

Cmm dilution	Plate count (Undiluted; 100 µL/plate)			Plate count (Diluted 1.4 µL/mL buffer; 100 µL/plate)			Cmm conc. (Avg. CFU/1.4 µL undiluted dilution)
	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3	
1	n/d	n/d	n/d	66	76	63	680
2	n/d	n/d	n/d	14	10	16	136
3	n/d	n/d	n/d	1	1	1	27.2
4	333	337	334	1	0	0	5.4
5	47	65	58	1	0	0	1.1
6	0	0	0	n/d	n/d	n/d	0

Table C2. Dilution plating results of test samples for validating analytical sensitivity (§3.2).
Extract prepared from four different seed batches was spiked with a five-fold dilution series of liquid cultured Cmm (dilutions 1-5; 680, 136, 27.2, 5.4 and 1.1 CFU/mL seed extract). Dilution 6 did not contain any Cmm (negative control). Each Cmm dilution was spiked to four subsamples of each seed batch. The extract was plated non-concentrated, and 10-fold concentrated on Cmm1T and FSCM media plates. Cmm colonies and saprophytes were scored for each plate.

Sample (Seed batch_Cmm dilution, Rep.)	Cmm				Saprophytes			
	Cmm1T		FSCM		Cmm1T		FSCM	
	Non-conc.	10x conc.	Non-conc.	10x conc.	Non-conc.	10x conc.	Non-conc.	10x conc.
1_1,1	58	616	65	428				
1_1,2	84	556	75	416				
1_1,3	53	476	53	504				
1_1,4	73	580	64	468	1			
1_2,1	14	153	20	136				
1_2,2	12	142	9	109	1			
1_2,3	19	136	8	141				
1_2,4	13	143	14	146	1	3		
1_3,1	3	32	9	27				
1_3,2	5	26	3	27			1	
1_3,3	0	32	2	26	2	1		
1_3,4	3	20	0	27		3		

Sample (Seed batch_Cmm dilution, Rep.)	Cmm				Saprophytes			
	Cmm1T		FSCM		Cmm1T		FSCM	
	Non-conc.	10x conc.	Non-conc.	10x conc.	Non-conc.	10x conc.	Non-conc.	10x conc.
1_4,1	2	5	0	4				
1_4,2	0	8	0	9		3		
1_4,3	0	11	1	9		3		
1_4,4	0	3	0	10		1	1	
1_5,1	0	2	0	0	1	1		
1_5,2	0	1	0	5	2			
1_5,3	1	1	0	1				
1_5,4	0	0	0	0	1	17		
1_6,1	0	0	0	0		2		
1_6,2	0	0	0	0		2		1
1_6,3	0	0	0	0				
1_6,4	0	0	0	0	1	26	82	+++
2_1,1	66	720	51	676				
2_1,2	41	900	60	620				
2_1,3	77	884	66	468				
2_1,4	68	652	55	612				
2_2,1	13	184	12	147				
2_2,2	8	150	17	181				
2_2,3	17	180	7	190				
2_2,4	15	163	10	183				
2_3,1	0	26	1	36				
2_3,2	9	43	1	47		2		
2_3,3	2	44	1	36				
2_3,4	5	39	2	28				
2_4,1	0	8	1	6	2	26		
2_4,2	1	8	0	3	5			34
2_4,3	0	13	0	6		2		
2_4,4	0	10	0	3	1			
2_5,1	0	2	0	0				
2_5,2	0	3	0	0		2		
2_5,3	0	2	0	0				
2_5,4	2	3	1	1				
2_6,1	0	0	0	0				
2_6,2	0	0	0	0		4		
2_6,3	0	0	0	0				
2_6,4	0	0	0	0				
3_1,1	66	360	56	320				
3_1,2	63	748	74	520	1			

Sample (Seed batch_Cmm dilution, Rep.)	Cmm				Saprophytes			
	Cmm1T		FSCM		Cmm1T		FSCM	
	Non-conc.	10x conc.	Non-conc.	10x conc.	Non-conc.	10x conc.	Non-conc.	10x conc.
3_1,3	44	672	56	500	3			
3_1,4	50	680	71	440	2			
3_2,1	12	161	17	142		1		
3_2,2	13	154	11	145	4	24		
3_2,3	7	142	7	114	1	25		
3_2,4	9	151	9	151	3	2		
3_3,1	1	21	6	42	1	3		
3_3,2	6	28	2	23	11	65		
3_3,3	3	43	5	20		5		
3_3,4	2	31	2	31		8		
3_4,1	0	7	0	6	4	24		
3_4,2	1	9	1	10	1	23		
3_4,3	0	5	0	5				
3_4,4	1	8	0	7		5		
3_5,1	0	0	0	0	1	7		
3_5,2	0	0	0	2	1	1		
3_5,3	0	1	0	3		2		
3_5,4	0	0	0	2		5		
3_6,1	0	0	0	0	2	5		
3_6,2	0	0	0	0		8		
3_6,3	0	0	0	0		1		
3_6,4	0	0	0	0	1	3		
4_1,1	51	600	65	644				
4_1,2	70	532	57	512				
4_1,3	54	624	52	456				
4_1,4	53	616	49	488	2			
4_2,1	12	115	15	167	2	8		
4_2,2	19	122	12	153		4		
4_2,3	11	156	7	108	1	1		
4_2,4	17	118	9	154	1			
4_3,1	1	19	2	16		1		
4_3,2	2	27	0	24		1		
4_3,3	1	29	3	25		2		
4_3,4	0	25	2	18		1		
4_4,1	0	6	1	5		4		
4_4,2	0	5	0	10		3		
4_4,3	0	8	0	8				
4_4,4	0	6	0	14		1		

Sample (Seed batch_Cmm dilution, Rep.)	Cmm				Saprophytes			
	Cmm1T		FSCM		Cmm1T		FSCM	
	Non-conc.	10x conc.	Non-conc.	10x conc.	Non-conc.	10x conc.	Non-conc.	10x conc.
4_5,1	0	0	0	2	1	2		
4_5,2	0	0	0	0				
4_5,3	0	4	0	1		2		
4_5,4	0	1	0	0	1	2		
4_6,1	0	0	0	0		1		
4_6,2	0	0	0	0		1	1	
4_6,3	0	0	0	0		1		
4_6,4	0	0	0	0				

Table C3. SE-qPCR results of test samples for validating analytical sensitivity, selectivity, and repeatability (§3.2, 3.3 and 3.4).
Extract prepared from four different seed batches was spiked with a five-fold dilution series of liquid cultured Cmm (dilutions 1-5; 680, 136, 27.2, 5.4 and 1.1 CFU/mL seed extract). Dilution 6 did not contain any Cmm (negative control). Each Cmm dilution was spiked to four subsamples of each seed batch. The extract was processed according to the ISHI Cmm SE-qPCR protocol (Annex A). Shown are Cq results for the two qPCR assays detecting Cmm (PTSSK and MVS21+) and the assay detecting the Cmt internal control. Results were generated in two separate PCR runs. Positive amplification control samples (PACs) for Cmm and Cmt and Negative Template Control samples (NTCs) were included in each run.

Sample (Seed batch_Cmm dilution, Rep.)	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)	PCR plate
1_1,1	26.43	27.64	29.22	1
1_1,2	26.09	27.49	28.83	1
1_1,3	26.04	27.27	28.75	1
1_1,4	26.19	27.40	28.92	1
1_2,1	28.85	30.07	29.04	1
1_2,2	28.87	30.44	29.40	1
1_2,3	28.54	29.83	29.10	1
1_2,4	28.52	29.98	28.89	1
1_3,1	31.01	32.10	28.83	1
1_3,2	30.45	32.02	28.18	1
1_3,3	30.86	31.28	28.43	1
1_3,4	30.25	31.97	28.32	1
1_4,1	33.04	33.72	28.73	1
1_4,2	33.38	34.94	28.36	1
1_4,3	32.47	34.47	28.58	1
1_4,4	33.30	34.96	28.41	1
1_5,1	34.93	36.92	28.46	1
1_5,2	40.00	35.38	28.38	1
1_5,3	35.42	35.69	28.22	1
1_5,4	34.68	37.20	28.58	1
1_6,1	40.00	40.00	28.61	1
1_6,2	40.00	40.00	28.77	1
1_6,3	40.00	40.00	28.34	1
1_6,4	40.00	40.00	28.64	1
2_1,1	26.22	27.40	29.03	1
2_1,2	26.61	28.04	29.49	1
2_1,3	26.11	27.54	29.13	1
2_1,4	26.18	27.55	29.19	1
2_2,1	28.65	30.35	30.04	1
2_2,2	28.56	30.10	29.27	1

Sample (Seed batch_Cmm dilution, Rep.)	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)	PCR plate
2_2,3	28.88	30.09	29.53	1
2_2,4	29.02	30.47	29.61	1
2_3,1	31.15	32.43	29.75	1
2_3,2	30.70	32.00	29.00	1
2_3,3	31.06	32.17	29.27	1
2_3,4	30.88	31.63	29.06	1
2_4,1	33.76	35.20	29.18	1
2_4,2	33.69	34.56	29.33	1
2_4,3	33.18	34.97	28.83	1
2_4,4	35.11	34.69	29.45	1
2_5,1	34.35	37.12	29.33	1
2_5,2	35.84	40.00	28.88	1
2_5,3	34.15	36.28	29.32	1
2_5,4	40.00	35.81	29.19	1
2_6,1	40.00	40.00	29.19	1
2_6,2	40.00	40.00	28.88	1
2_6,3	40.00	40.00	29.75	1
2_6,4	40.00	40.00	29.29	1
3_1,1	26.53	28.58	29.34	2
3_1,2	26.52	28.21	28.88	2
3_1,3	27.16	28.88	29.41	2
3_1,4	26.71	28.44	29.25	2
3_2,1	29.35	30.83	29.81	2
3_2,2	28.67	30.60	29.34	2
3_2,3	28.94	31.09	29.38	2
3_2,4	28.87	30.57	29.30	2
3_3,1	31.18	32.78	29.02	2
3_3,2	30.60	32.44	29.02	2
3_3,3	31.32	33.19	28.83	2
3_3,4	31.71	33.44	29.40	2
3_4,1	35.00	35.05	29.91	2
3_4,2	33.46	34.41	29.40	2
3_4,3	34.99	34.96	29.43	2
3_4,4	34.27	36.93	29.51	2
3_5,1	40.00	40.00	29.10	2
3_5,2	35.71	40.00	28.99	2
3_5,3	36.23	40.00	29.24	2
3_5,4	35.02	37.19	29.30	2
3_6,1	40.00	40.00	29.98	2

Sample (Seed batch_Cmm dilution, Rep.)	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)	PCR plate
3_6,2	40.00	40.00	29.11	2
3_6,3	40.00	40.00	29.29	2
3_6,4	40.00	40.00	29.11	2
4_1,1	26.52	28.42	29.49	2
4_1,2	26.08	27.98	29.03	2
4_1,3	26.50	28.27	29.34	2
4_1,4	26.10	27.94	28.92	2
4_2,1	28.54	30.43	29.26	2
4_2,2	28.41	30.39	29.14	2
4_2,3	28.53	30.78	29.46	2
4_2,4	28.94	30.48	29.66	2
4_3,1	31.06	32.38	29.23	2
4_3,2	30.38	32.44	29.09	2
4_3,3	30.79	32.47	29.12	2
4_3,4	30.52	32.71	29.25	2
4_4,1	33.04	34.14	28.92	2
4_4,2	32.94	34.33	29.01	2
4_4,3	33.53	34.78	28.87	2
4_4,4	32.57	34.68	28.92	2
4_5,1	35.19	36.29	29.08	2
4_5,2	34.76	35.87	29.02	2
4_5,3	34.19	39.16	28.85	2
4_5,4	40.00	40.00	29.02	2
4_6,1	40.00	40.00	28.95	2
4_6,2	40.00	40.00	28.89	2
4_6,3	40.00	40.00	28.94	2
4_6,4	40.00	40.00	29.25	2
PAC Cmm	26.20	27.18	40.00	1
PAC Cmm	26.15	27.04	40.00	1
PAC Cmt	40.00	40.00	25.19	1
PAC Cmt	40.00	40.00	25.23	1
NTC	40.00	40.00	40.00	1
NTC	40.00	40.00	40.00	1
PAC Cmm	26.18	27.38	40.00	2
PAC Cmm	26.14	27.34	40.00	2
PAC Cmt	40.00	40.00	25.14	2
PAC Cmt	40.00	40.00	25.11	2
NTC	40.00	40.00	40.00	2
NTC	40.00	40.00	40.00	2

Table C4. Dilution plating results of test samples for validating diagnostic performance (§3.6). A total of 50 subsamples of 10,000 seeds from a healthy seed lot were spiked with a single seed harvested from Cmm-infected tomatoes. 10 subsamples were not spiked and processed as NPCs. The extract was plated non-concentrated, 10-fold concentrated, and 10-fold diluted on Cmm1T and FSCM media plates (100 µL/plate). Plates were scored for Cmm colonies after eight days of incubation at 27 °C. Legend: n/d is not determined.

Sample	Cmm1T			FSCM			Average CFU/mL non-concentrated seed extract
	10-fold conc.	Non-conc.	10-fold dil.	10-fold conc.	Non-conc.	10-fold dil.	
1	18	n/d	n/d	32	n/d	n/d	25
2	15	n/d	n/d	17	n/d	n/d	16
3	n/d	33	n/d	n/d	34	n/d	335
4	n/d	130	25	n/d	n/d	58	3550
5	25	n/d	n/d	47	n/d	n/d	36
6	30	n/d	n/d	51	n/d	n/d	40.5
7	n/d	160	n/d	n/d	53	n/d	1065
8	n/d	n/d	51	n/d	n/d	88	6950
9	40	n/d	n/d	59	n/d	n/d	49.5
10	n/d	n/d	58	n/d	n/d	61	5950
11	n/d	n/d	246	n/d	n/d	243	24450
12	n/d	214	19	n/d	190	n/d	2020
13	203	33	n/d	n/d	47	n/d	400
14	n/d	98	n/d	n/d	77	n/d	875
15	12	n/d	n/d	13	n/d	n/d	12.5
16	n/d	n/d	75	n/d	510	0	6300
17	40	n/d	n/d	0	4	n/d	40
18	29	n/d	n/d	58	n/d	n/d	43.5
19	23	n/d	n/d	35	n/d	n/d	29
20	53	n/d	n/d	79	n/d	n/d	66
21	n/d	n/d	39	n/d	n/d	32	3550
22	n/d	38	n/d	n/d	53	n/d	455
23	n/d	n/d	159	n/d	n/d	133	14600
24	n/d	n/d	144	n/d	n/d	176	16000
25	136	25	n/d	150	37	n/d	200
26	39	n/d	n/d	42	n/d	n/d	40.5
27	n/d	n/d	136	n/d	n/d	129	13250
28	n/d	n/d	92	n/d	n/d	113	10250
29	n/d	41	n/d	n/d	40	n/d	405
30	n/d	n/d	59	n/d	n/d	66	6250
31	n/d	215	26	n/d	n/d	67	4425
32	169	30	n/d	142	36	n/d	330
33	107	20	n/d	122	20	n/d	200
34	n/d	n/d	171	n/d	n/d	171	17100

Sample	Cmm1T			FSCM			Average CFU/mL non-concentrated seed extract
	10-fold conc.	Non-conc.	10-fold dil.	10-fold conc.	Non-conc.	10-fold dil.	
35	n/d	n/d	236	n/d	n/d	270	25300
36	73	n/d	n/d	98	n/d	n/d	85.5
37	n/d	n/d	89	n/d	n/d	96	9250
38	n/d	56	n/d	n/d	37	n/d	465
39	n/d	n/d	155	n/d	n/d	116	13550
40	58	n/d	n/d	n/d	n/d	n/d	59.5
41	n/d	n/d	97	n/d	n/d	174	13550
42	40	n/d	n/d	42	n/d	n/d	41
43	79	n/d	n/d	48	n/d	n/d	63.5
44	n/d	n/d	62	n/d	n/d	14	3800
45	43	n/d	n/d	n/d	n/d	n/d	47.5
46	n/d	n/d	221	n/d	n/d	171	19600
47	n/d	n/d	134	n/d	n/d	118	12600
48	100	n/d	n/d	95	n/d	n/d	97.5
49	35	n/d	n/d	37	n/d	n/d	36
50	n/d	n/d	111	n/d	n/d	85	9800
51 (NPC)	0	n/d	n/d	0	n/d	n/d	0
52 (NPC)	0	n/d	n/d	0	n/d	n/d	0
53 (NPC)	0	n/d	n/d	0	n/d	n/d	0
54 (NPC)	0	n/d	n/d	0	n/d	n/d	0
55 (NPC)	0	n/d	n/d	0	n/d	n/d	0
56 (NPC)	0	n/d	n/d	0	n/d	n/d	0
57 (NPC)	0	n/d	n/d	0	n/d	n/d	0
58 (NPC)	0	n/d	n/d	0	n/d	n/d	0
59 (NPC)	0	n/d	n/d	0	n/d	n/d	0
60 (NPC)	0	n/d	n/d	0	n/d	n/d	0

Table C5. SE-qPCR results of test samples for validating diagnostic performance (§3.6).
A total of 50 subsamples of 10,000 seeds from a healthy seed lot were spiked with a single seed harvested from Cmm-infected tomatoes. A total of 10 subsamples were not spiked and processed as NPCs. The extract was processed according to the ISHI Cmm SE-qPCR protocol (Annex A). Shown are Cq results for the two qPCR assays detecting Cmm (PTSSK and MVS21+) and the assay detecting the Cmt internal control. Results were generated in two separate PCR runs. Positive amplification control samples (PACs) for Cmm and Cmt and Negative template control samples (NTCs) were included in each run.

Sample	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)	PCR plate
1	25.04	25.59	28.65	1
2	25.23	26.02	28.63	1
3	23.58	24.57	28.27	1
4	22.03	22.85	28.23	1
5	25.20	26.08	28.59	1
6	25.07	25.72	28.28	1
7	24.17	25.10	28.94	1
8	22.04	22.72	28.86	1
9	25.09	25.74	28.84	1
10	21.02	21.76	28.03	1
11	21.19	22.30	28.79	1
12	20.30	21.31	29.11	1
13	23.95	24.75	29.10	1
14	22.12	22.95	28.62	1
15	26.01	26.67	29.28	1
16	21.14	22.17	29.01	1
17	25.38	26.09	29.34	1
18	25.38	26.29	29.14	1
19	25.41	26.27	28.72	1
20	23.49	24.52	28.80	1
21	22.18	23.38	28.45	1
22	23.02	23.63	28.78	1
23	20.76	21.48	28.29	1
24	19.97	20.57	28.75	1
25	23.50	24.46	28.30	1
26	25.05	25.73	28.79	1
27	20.27	21.08	28.39	1
28	21.01	21.74	28.45	1
29	23.48	25.38	28.41	1
30	20.37	21.41	28.19	1
31	21.18	22.20	28.40	1
32	24.36	25.33	28.68	1

Sample	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)	PCR plate
33	24.31	25.23	28.82	1
34	19.85	20.52	28.16	1
35	20.03	20.80	28.27	1
36	25.12	25.83	28.80	1
37	20.88	21.58	28.63	1
38	23.46	24.45	28.39	1
39	19.88	20.61	28.52	1
40	23.49	24.47	28.56	1
41	21.10	22.14	29.09	2
42	25.01	26.14	28.88	2
43	21.74	22.85	28.22	2
44	21.32	22.66	29.09	2
45	24.27	25.57	29.12	2
46	21.04	22.24	29.26	2
47	19.28	20.59	28.32	2
48	23.62	25.03	28.77	2
49	24.76	26.12	28.74	2
50	20.90	22.04	28.33	2
51 (NPC)	40.00	40.00	29.28	2
52 (NPC)	40.00	40.00	29.68	2
53 (NPC)	40.00	40.00	28.56	2
54 (NPC)	40.00	40.00	28.66	2
55 (NPC)	40.00	40.00	28.69	2
56 (NPC)	40.00	40.00	28.75	2
57 (NPC)	40.00	40.00	28.87	2
58 (NPC)	36.48	38.03	28.79	2
59 (NPC)	36.57	40.00	28.62	2
60 (NPC)	40.00	38.49	29.06	2
PAC Cmm	31.77	31.54	40.00	1
PAC Cmm	32.14	31.96	40.00	1
PAC Cmt	40.00	40.00	26.48	1
PAC Cmt	40.00	40.00	26.08	1
NTC	40.00	40.00	40.00	1
NTC	40.00	40.00	40.00	1
PAC Cmm	31.96	32.10	40.00	2
PAC Cmm	31.38	31.50	40.00	2
PAC Cmt	40.00	40.00	26.73	2
PAC Cmt	40.00	40.00	26.79	2
NTC	40.00	40.00	40.00	2

Sample	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)	PCR plate
NTC	40.00	40.00	40.00	2

Table C6. Homogeneity results of CT test samples for validating reproducibility (§3.5; Annex B).
Prior to supplying participants with seed material, 10 subsamples of 10,000 seeds from each of the 6 seed backgrounds included in the CT (see Table B1 for background compositions) were processed by SE-qPCR (Annex A). Shown are Cq results for the two qPCR assays detecting Cmm (PTSSK and MVS21+) and the assay detecting the Cmt internal control. Results were generated in two separate PCR runs. Positive amplification control samples (PACs) for Cmm and Cmt and Negative template control samples (NTCs) were included in each run.

Seed background_subsample	Cmm infection level	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)	PCR plate
A1	Healthy	40.00	40.00	28.50	1
A2	Healthy	40.00	40.00	28.78	1
A3	Healthy	40.00	40.00	28.69	1
A4	Healthy	40.00	40.00	28.66	1
A5	Healthy	40.00	40.00	28.53	1
A6	Healthy	40.00	40.00	28.71	1
A7	Healthy	40.00	40.00	29.16	1
A8	Healthy	40.00	40.00	29.17	1
A9	Healthy	40.00	40.00	29.10	1
A10	Healthy	40.00	40.00	29.07	1
B1	Low	30.29	29.67	28.72	1
B2	Low	30.46	29.88	28.91	1
B3	Low	30.24	29.68	29.19	1
B4	Low	30.25	29.63	28.99	1
B5	Low	30.19	29.88	28.63	1
B6	Low	29.54	29.13	28.30	1
B7	Low	29.96	29.65	28.45	1
B8	Low	30.23	29.45	28.60	1
B9	Low	30.12	29.53	28.39	1
B10	Low	30.45	29.60	28.65	1
C1	Low	30.07	30.35	28.79	1
C2	Low	30.55	31.03	29.04	1
C3	Low	31.19	32.09	28.44	1
C4	Low	30.71	31.84	29.27	1
C5	Low	30.69	31.22	29.14	1
C6	Low	30.80	31.33	29.07	1
C7	Low	30.47	31.70	28.70	1
C8	Low	30.85	31.71	29.38	1

Seed background_subsample	Cmm infection level	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)	PCR plate
C9	Low	30.29	31.03	28.79	1
C10	Low	30.43	31.09	28.60	1
D1	Medium	28.26	27.90	28.15	1
D2	Medium	29.27	28.81	28.65	1
D3	Medium	29.37	28.76	28.57	1
D4	Medium	23.30	23.00	28.10	1
D5	Medium	29.24	28.56	28.12	1
D6	Medium	29.71	29.61	28.43	2
D7	Medium	29.12	28.62	28.73	2
D8	Medium	27.99	27.29	28.50	2
D9	Medium	29.23	28.90	28.78	2
D10	Medium	29.46	29.21	28.51	2
E1	Medium	27.76	28.63	29.40	2
E2	Medium	27.52	28.26	28.66	2
E3	Medium	27.35	28.22	29.03	2
E4	Medium	27.30	28.17	29.04	2
E5	Medium	27.33	28.15	29.24	2
E6	Medium	27.02	27.93	28.78	2
E7	Medium	27.12	27.80	28.97	2
E8	Medium	27.29	27.97	29.07	2
E9	Medium	27.30	28.13	28.25	2
E10	Medium	27.86	28.35	29.59	2
F1	High	24.19	24.04	29.13	2
F2	High	25.29	25.19	29.13	2
F3	High	25.18	25.07	29.15	2
F4	High	25.56	25.43	28.66	2
F5	High	25.31	25.08	29.17	2
F6	High	24.55	24.37	28.76	2
F7	High	25.68	25.70	29.27	2
F8	High	25.51	25.60	29.10	2
F9	High	25.98	25.68	29.33	2
F10	High	25.23	25.07	28.51	2
PAC Cmm	-	31.33	31.30	40.00	1
PAC Cmm	-	31.69	31.40	40.00	1
PAC Cmt	-	40.00	40.00	25.27	1
PAC Cmt	-	40.00	40.00	25.32	1
NTC	-	40.00	40.00	40.00	1
NTC	-	40.00	40.00	40.00	1
PAC Cmm	-	31.22	31.16	40.00	2

Seed background_subsample	Cmm infection level	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)	PCR plate
PAC Cmm	-	32.35	32.09	40.00	2
PAC Cmt	-	40.00	40.00	25.31	2
PAC Cmt	-	40.00	40.00	25.25	2
NTC	-	40.00	40.00	40.00	2
NTC	-	40.00	40.00	40.00	2

Table C7. Stability results of CT test samples for validating reproducibility (§3.5; Annex B). After the CT was completed by all participants, 10 subsamples of 10,000 seeds from each of the six seed backgrounds included in the CT (see Table B1 for background compositions) were processed by SE-qPCR (Annex A). Shown are Cq results for the two qPCR assays detecting Cmm (PTSSK and MVS21+) and the assay detecting the Cmt internal control. Results were generated in two separate PCR runs. Positive amplification control samples (PACs) for Cmm and Cmt and Negative template control samples (NTCs) were included in each run.

Seed background _subsample	Cmm infection level	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)	PCR plate
A1	Healthy	40.00	40.00	29.79	1
A2	Healthy	40.00	40.00	29.72	1
A3	Healthy	40.00	40.00	29.45	1
A4	Healthy	40.00	40.00	29.41	1
A5	Healthy	40.00	40.00	29.65	1
A6	Healthy	40.00	40.00	29.38	1
A7	Healthy	40.00	40.00	29.85	1
A8	Healthy	40.00	40.00	30.01	1
A9	Healthy	40.00	40.00	29.47	1
A10	Healthy	40.00	40.00	29.11	1
B1	Low	31.08	30.06	28.90	1
B2	Low	31.03	30.09	28.69	1
B3	Low	30.96	30.09	28.89	1
B4	Low	31.09	30.19	29.11	1
B5	Low	30.69	30.03	28.60	1
B6	Low	30.81	30.25	28.86	1
B7	Low	31.28	30.68	29.38	1
B8	Low	30.94	30.22	28.78	1
B9	Low	31.20	30.31	29.34	1
B10	Low	31.51	30.70	29.40	1
C1	Low	31.92	31.85	28.82	1
C2	Low	30.73	31.26	28.56	1
C3	Low	31.01	31.08	29.10	1
C4	Low	31.29	31.64	28.33	1
C5	Low	31.98	31.27	29.47	1
C6	Low	31.57	32.49	28.72	1
C7	Low	31.39	31.31	29.16	1
C8	Low	31.40	31.45	29.43	1
C9	Low	31.74	31.73	29.78	1
C10	Low	31.54	31.97	29.94	1
D1	Medium	29.43	28.78	29.57	1
D2	Medium	29.46	28.74	30.39	1
D3	Medium	29.83	29.45	29.37	2

Seed background _subsample	Cmm infection level	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)	PCR plate
D4	Medium	29.75	29.33	29.17	2
D5	Medium	29.92	29.70	29.32	2
D6	Medium	29.49	29.16	29.35	2
D7	Medium	30.03	29.72	29.38	2
D8	Medium	29.57	29.31	29.26	2
D9	Medium	29.56	29.22	29.13	2
D10	Medium	29.89	29.32	29.06	2
E1	Medium	28.02	28.54	30.05	2
E2	Medium	27.50	28.07	28.99	2
E3	Medium	28.26	28.84	29.53	2
E4	Medium	28.00	28.38	29.02	2
E5	Medium	27.66	28.42	29.29	2
E6	Medium	27.79	28.19	29.45	2
E7	Medium	28.26	28.68	29.46	2
E8	Medium	27.97	28.47	29.93	2
E9	Medium	27.92	28.49	29.97	2
E10	Medium	28.23	28.65	29.35	2
F1	High	26.56	26.13	29.82	2
F2	High	25.80	25.52	29.43	2
F3	High	25.82	25.23	29.31	2
F4	High	26.01	25.29	29.35	2
F5	High	25.37	25.09	29.05	2
F6	High	25.84	25.29	29.86	2
F7	High	26.29	25.87	30.53	2
F8	High	26.22	25.74	30.38	2
F9	High	25.56	25.17	29.94	2
F10	High	26.16	25.67	30.16	2
PAC Cmm	-	31.50	30.60	40.00	1
PAC Cmm	-	31.41	30.85	40.00	1
PAC Cmt	-	40.00	40.00	28.94	1
PAC Cmt	-	40.00	40.00	29.22	1
NTC	-	40.00	40.00	40.00	1
NTC	-	40.00	40.00	40.00	1
PAC Cmm	-	32.32	32.36	40.00	2
PAC Cmm	-	31.08	31.13	40.00	2
PAC Cmt	-	40.00	40.00	29.23	2
PAC Cmt	-	40.00	40.00	29.27	2
NTC	-	40.00	40.00	40.00	2
NTC	-	40.00	40.00	40.00	2

Table C8. Raw CT test results. Three subsamples of six seed backgrounds were processed by SE- qPCR (Annex A) by seven participating labs (organizing lab excluded).
Backgrounds varied in Cmm-infection (healthy/low/medium/high). DNA isolated from subsamples was analysed by qPCR in technical duplicates. Shown are Cq results for the two qPCR assays detecting Cmm (PTSSK and MVS21+) and the assay detecting the Cmt internal control. Positive amplification control samples (PACs) for Cmm and Cmt and Negative Template Control samples (NTCs) were included. Participants scored the health status of each subsample (Cmm negative or suspect).

Lab#	Sample code	Seed lot	Cmm infection level	PCR duplicate	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)	Cmm validation
1	1	C	Low	A	31.36	32.40	23.61	Suspect
1	1	C	Low	B	32.01	31.62	23.42	Suspect
1	2	C	Low	A	31.63	31.86	23.42	Suspect
1	2	C	Low	B	31.69	32.24	23.84	Suspect
1	3	E	Medium	A	28.91	29.28	24.85	Suspect
1	3	E	Medium	B	28.58	29.21	24.84	Suspect
1	4	B	Low	A	30.31	30.01	23.62	Suspect
1	4	B	Low	B	30.63	30.32	23.88	Suspect
1	5	F	High	A	28.74	28.08	25.79	Suspect
1	5	F	High	B	28.96	28.28	25.95	Suspect
1	6	E	Medium	A	29.02	29.18	24.99	Suspect
1	6	E	Medium	B	29.01	29.49	25.27	Suspect
1	7	B	Low	A	30.70	30.45	25.08	Suspect
1	7	B	Low	B	30.97	30.42	25.22	Suspect
1	8	A	Healthy	A	40.00	40.00	24.78	Negative
1	8	A	Healthy	B	40.00	38.16	24.92	Negative
1	9	F	High	A	27.47	27.00	25.12	Suspect
1	9	F	High	B	27.45	26.99	25.02	Suspect
1	10	D	Medium	A	30.36	29.54	24.56	Suspect
1	10	D	Medium	B	30.47	29.75	25.19	Suspect
1	11	D	Medium	A	31.38	30.59	26.39	Suspect
1	11	D	Medium	B	31.21	30.14	26.17	Suspect
1	12	A	Healthy	A	40.00	37.45	23.74	Negative
1	12	A	Healthy	B	40.00	40.00	24.99	Negative
1	13	A	Healthy	A	40.00	40.00	24.77	Negative
1	13	A	Healthy	B	40.00	40.00	24.62	Negative
1	14	C	Low	A	33.30	33.88	25.22	Suspect
1	14	C	Low	B	33.42	33.43	24.94	Suspect
1	15	F	High	A	26.47	26.54	26.20	Suspect
1	15	F	High	B	26.63	26.60	26.03	Suspect
1	16	D	Medium	A	29.65	28.99	25.04	Suspect
1	16	D	Medium	B	30.09	29.46	25.55	Suspect
1	17	B	Low	A	30.56	30.77	25.28	Suspect

Lab#	Sample code	Seed lot	Cmm infection level	PCR duplicate	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)	Cmm validation
1	17	B	Low	B	30.98	30.77	25.28	Suspect
1	18	E	Medium	A	27.36	26.85	24.20	Suspect
1	18	E	Medium	B	27.93	28.83	25.74	Suspect
1	PAC Cmm	-	-	A	20.89	22.26	40.00	-
1	PAC Cmm	-	-	B	20.85	22.32	40.00	-
1	PAC IAC	-	-	A	40.00	40.00	18.95	-
1	PAC IAC	-	-	B	40.00	40.00	18.79	-
1	NTC	-	-	A	40.00	40.00	40.00	-
1	NTC	-	-	B	40.00	40.00	40.00	-
2	1	C	Low	A	33.74	32.67	29.35	Positive
2	1	C	Low	B	33.77	33.67	29.27	Positive
2	2	C	Low	A	32.66	32.21	28.96	Positive
2	2	C	Low	B	33.36	32.71	28.82	Positive
2	3	E	Medium	A	31.28	30.86	29.17	Positive
2	3	E	Medium	B	31.86	30.96	29.24	Positive
2	4	B	Low	A	31.98	30.27	28.96	Positive
2	4	B	Low	B	31.98	30.12	28.78	Positive
2	5	F	High	A	29.58	29.11	28.86	Positive
2	5	F	High	B	29.99	29.18	29.03	Positive
2	6	E	Medium	A	27.80	26.26	28.98	Positive
2	6	E	Medium	B	28.63	26.75	28.86	Positive
2	7	B	Low	A	31.68	29.71	27.76	Positive
2	7	B	Low	B	31.76	30.01	27.83	Positive
2	8	A	Healthy	A	36.55	40.00	28.61	Negative
2	8	A	Healthy	B	36.24	40.00	28.58	Negative
2	9	F	High	A	28.23	26.71	28.44	Positive
2	9	F	High	B	28.23	26.68	28.38	Positive
2	10	D	Medium	A	30.64	28.98	28.01	Positive
2	10	D	Medium	B	30.78	29.14	28.15	Positive
2	11	D	Medium	A	31.11	29.48	28.24	Positive
2	11	D	Medium	B	31.03	29.41	28.69	Positive
2	12	A	Healthy	A	40.00	40.00	29.59	Negative
2	12	A	Healthy	B	40.00	40.00	29.58	Negative
2	13	A	Healthy	A	40.00	40.00	29.15	Negative
2	13	A	Healthy	B	40.00	38.11	29.34	Negative
2	14	C	Low	A	32.35	32.28	28.79	Positive
2	14	C	Low	B	32.83	32.63	29.01	Positive
2	15	F	High	A	27.09	25.65	28.11	Positive
2	15	F	High	B	26.89	25.62	28.18	Positive

Lab#	Sample code	Seed lot	Cmm infection level	PCR duplicate	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)	Cmm validation
2	16	D	Medium	A	31.21	29.81	28.83	Positive
2	16	D	Medium	B	31.00	29.82	28.83	Positive
2	17	B	Low	A	32.16	30.12	28.86	Positive
2	17	B	Low	B	32.24	30.31	28.73	Positive
2	18	E	Medium	A	29.93	29.73	29.56	Positive
2	18	E	Medium	B	32.13	32.18	31.26	Positive
2	PAC Cmm	-	-	A	27.06	27.54	38.33	-
2	PAC Cmm	-	-	B	26.52	27.61	38.39	-
2	PAC IAC	-	-	A	40.00	40.00	29.45	-
2	PAC IAC	-	-	B	40.00	40.00	29.66	-
2	NTC	-	-	A	40.00	40.00	40.00	-
2	NTC	-	-	B	40.00	40.00	40.00	-
3	1	C	Low	A	32.46	33.07	29.70	Suspect
3	1	C	Low	B	32.79	33.46	29.48	Suspect
3	2	C	Low	A	32.72	33.20	29.61	Suspect
3	2	C	Low	B	32.92	33.24	29.43	Suspect
3	3	E	Medium	A	29.27	29.65	29.91	Suspect
3	3	E	Medium	B	29.37	29.72	29.92	Suspect
3	4	B	Low	A	32.96	31.60	30.00	Suspect
3	4	B	Low	B	32.60	31.94	30.17	Suspect
3	5	F	High	A	27.84	27.21	30.54	Suspect
3	5	F	High	B	28.27	27.07	30.78	Suspect
3	6	E	Medium	A	29.37	29.70	29.62	Suspect
3	6	E	Medium	B	29.48	29.72	29.82	Suspect
3	7	B	Low	A	32.27	31.90	29.94	Suspect
3	7	B	Low	B	32.49	31.69	29.94	Suspect
3	8	A	Healthy	A	40.00	40.00	29.88	Negative
3	8	A	Healthy	B	40.00	40.00	29.74	Negative
3	9	F	High	A	27.68	26.81	30.54	Suspect
3	9	F	High	B	27.89	26.77	30.28	Suspect
3	10	D	Medium	A	31.19	30.50	29.34	Suspect
3	10	D	Medium	B	30.83	30.32	29.36	Suspect
3	11	D	Medium	A	30.98	29.99	29.37	Suspect
3	11	D	Medium	B	30.93	30.15	29.31	Suspect
3	12	A	Healthy	A	40.00	40.00	29.64	Negative
3	12	A	Healthy	B	38.46	40.00	29.60	Negative
3	13	A	Healthy	A	40.00	40.00	30.07	Negative
3	13	A	Healthy	B	40.00	40.00	29.76	Negative
3	14	C	Low	A	33.27	33.92	29.51	Suspect

Lab#	Sample code	Seed lot	Cmm infection level	PCR duplicate	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)	Cmm validation
3	14	C	Low	B	33.50	33.98	29.48	Suspect
3	15	F	High	A	27.90	27.14	30.72	Suspect
3	15	F	High	B	27.93	27.19	30.54	Suspect
3	16	D	Medium	A	31.52	30.40	29.91	Suspect
3	16	D	Medium	B	31.16	30.41	29.55	Suspect
3	17	B	Low	A	32.09	31.01	29.40	Suspect
3	17	B	Low	B	31.83	31.13	29.50	Suspect
3	18	E	Medium	A	29.05	29.41	29.86	Suspect
3	18	E	Medium	B	28.67	28.87	29.33	Suspect
3	PAC Cmm	-	-	A	28.26	29.27	40.00	-
3	PAC Cmm	-	-	B	27.86	29.11	40.00	-
3	PAC IAC	-	-	A	40.00	40.00	27.30	-
3	PAC IAC	-	-	B	40.00	40.00	27.32	-
3	NTC	-	-	A	40.00	40.00	40.00	-
3	NTC	-	-	B	40.00	40.00	40.00	-
4	1	C	Low	A	33.27	31.97	28.15	Suspect
4	1	C	Low	B	33.55	31.75	28.16	Suspect
4	2	C	Low	A	33.58	32.33	27.60	Suspect
4	2	C	Low	B	33.07	32.11	27.28	Suspect
4	3	E	Medium	A	29.17	27.49	28.05	Suspect
4	3	E	Medium	B	28.89	27.24	28.02	Suspect
4	4	B	Low	A	32.01	29.41	28.52	Suspect
4	4	B	Low	B	31.90	29.36	28.44	Suspect
4	5	F	High	A	28.55	26.06	28.89	Suspect
4	5	F	High	B	28.31	25.77	28.52	Suspect
4	6	E	Medium	A	29.12	27.38	28.05	Suspect
4	6	E	Medium	B	29.11	27.22	28.01	Suspect
4	7	B	Low	A	32.27	29.67	28.45	Suspect
4	7	B	Low	B	32.27	29.42	28.35	Suspect
4	8	A	Healthy	A	40.00	40.00	27.35	Negative
4	8	A	Healthy	B	40.00	37.90	27.57	Negative
4	9	F	High	A	28.18	25.61	28.61	Suspect
4	9	F	High	B	28.05	25.55	28.55	Suspect
4	10	D	Medium	A	31.01	28.39	28.18	Suspect
4	10	D	Medium	B	30.89	28.29	28.11	Suspect
4	11	D	Medium	A	30.83	28.23	27.65	Suspect
4	11	D	Medium	B	30.62	28.39	27.59	Suspect
4	12	A	Healthy	A	40.00	40.00	28.27	Negative
4	12	A	Healthy	B	40.00	40.00	28.21	Negative

Lab#	Sample code	Seed lot	Cmm infection level	PCR duplicate	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)	Cmm validation
4	13	A	Healthy	A	40.00	40.00	27.67	Negative
4	13	A	Healthy	B	40.00	40.00	27.50	Negative
4	14	C	Low	A	33.21	31.50	27.02	Suspect
4	14	C	Low	B	34.19	32.05	26.91	Suspect
4	15	F	High	A	27.66	25.12	28.39	Suspect
4	15	F	High	B	27.42	24.69	28.12	Suspect
4	16	D	Medium	A	30.69	28.08	27.57	Suspect
4	16	D	Medium	B	30.49	28.10	27.90	Suspect
4	17	B	Low	A	32.16	29.30	28.19	Suspect
4	17	B	Low	B	31.66	29.35	28.10	Suspect
4	18	E	Medium	A	29.62	28.10	28.10	Suspect
4	18	E	Medium	B	29.49	28.02	28.19	Suspect
4	PAC Cmm	-	-	A	30.47	29.69	27.72	-
4	PAC Cmm	-	-	B	30.44	29.59	27.82	-
4	PAC IAC	-	-	A	28.60	27.24	27.35	-
4	PAC IAC	-	-	B	28.64	27.07	27.23	-
4	NTC	-	-	A	40.00	40.00	30.49	-
4	NTC	-	-	B	40.00	40.00	27.29	-
5	1	C	Low	A	36.06	33.62	19.94	Suspect
5	1	C	Low	B	36.89	35.42	20.09	Suspect
5	2	C	Low	A	38.67	36.38	20.05	Negative
5	2	C	Low	B	38.65	36.01	19.95	Negative
5	3	E	Medium	A	31.57	30.95	20.92	Suspect
5	3	E	Medium	B	31.41	30.53	20.99	Suspect
5	4	B	Low	A	35.95	33.74	19.32	Suspect
5	4	B	Low	B	35.92	34.47	19.16	Suspect
5	5	F	High	A	28.63	26.02	21.65	Suspect
5	5	F	High	B	29.56	28.10	21.35	Suspect
5	6	E	Medium	A	31.10	29.45	20.11	Suspect
5	6	E	Medium	B	31.12	29.67	20.13	Suspect
5	7	B	Low	A	35.57	32.99	19.33	Suspect
5	7	B	Low	B	35.66	34.19	19.40	Suspect
5	8	A	Healthy	A	40.00	40.00	19.35	Negative
5	8	A	Healthy	B	40.00	40.00	19.29	Negative
5	9	F	High	A	29.69	28.06	19.05	Suspect
5	9	F	High	B	29.81	28.10	19.00	Suspect
5	10	D	Medium	A	35.09	33.17	18.67	Suspect
5	10	D	Medium	B	34.76	33.59	18.66	Suspect
5	11	D	Medium	A	34.46	32.97	18.66	Suspect

Lab#	Sample code	Seed lot	Cmm infection level	PCR duplicate	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)	Cmm validation
5	11	D	Medium	B	34.83	33.31	18.66	Suspect
5	12	A	Healthy	A	40.00	40.00	19.09	Negative
5	12	A	Healthy	B	40.00	40.00	19.04	Negative
5	13	A	Healthy	A	40.00	40.00	19.53	Negative
5	13	A	Healthy	B	40.00	40.00	19.23	Negative
5	14	C	Low	A	36.93	37.22	19.08	Negative
5	14	C	Low	B	36.03	36.03	18.86	Negative
5	15	F	High	A	30.26	28.77	20.63	Suspect
5	15	F	High	B	30.27	28.70	20.62	Suspect
5	16	D	Medium	A	36.01	34.01	21.39	Suspect
5	16	D	Medium	B	35.92	34.02	21.25	Suspect
5	17	B	Low	A	35.96	34.31	20.41	Suspect
5	17	B	Low	B	36.25	34.82	20.51	Suspect
5	18	E	Medium	A	31.94	28.84	21.06	Suspect
5	18	E	Medium	B	32.25	30.77	21.84	Suspect
5	PAC Cmm	-	-	A	18.25	18.67	40.00	-
5	PAC Cmm	-	-	B	18.01	18.00	40.00	-
5	PAC IAC	-	-	A	n/d	n/d	n/d	-
5	PAC IAC	-	-	B	n/d	n/d	n/d	-
5	NTC	-	-	A	40.00	40.00	40.00	-
5	NTC	-	-	B	40.00	40.00	40.00	-
6	1	C	Low	A	32.59	32.66	31.02	Suspect
6	1	C	Low	B	32.22	32.22	31.03	Suspect
6	2	C	Low	A	32.06	32.26	31.25	Suspect
6	2	C	Low	B	31.86	32.16	31.01	Suspect
6	3	E	Medium	A	29.81	28.76	30.49	Suspect
6	3	E	Medium	B	29.89	28.88	30.37	Suspect
6	4	B	Low	A	31.72	29.74	31.57	Suspect
6	4	B	Low	B	33.03	30.96	31.70	Suspect
6	5	F	High	A	28.48	25.93	31.13	Suspect
6	5	F	High	B	28.16	25.76	31.33	Suspect
6	6	E	Medium	A	29.91	28.87	31.20	Suspect
6	6	E	Medium	B	29.44	28.25	30.24	Suspect
6	7	B	Low	A	32.05	29.96	32.72	Suspect
6	7	B	Low	B	31.68	29.51	32.96	Suspect
6	8	A	Healthy	A	40.00	40.00	32.63	Negative
6	8	A	Healthy	B	40.00	40.00	32.80	Negative
6	9	F	High	A	28.75	26.23	33.19	Suspect
6	9	F	High	B	30.07	27.86	>35	Suspect

Lab#	Sample code	Seed lot	Cmm infection level	PCR duplicate	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)	Cmm validation
6	10	D	Medium	A	31.10	28.85	32.78	Suspect
6	10	D	Medium	B	31.23	29.11	33.25	Suspect
6	11	D	Medium	A	30.76	28.27	32.77	Suspect
6	11	D	Medium	B	30.72	28.19	32.76	Suspect
6	12	A	Healthy	A	>35	>35	32.46	Negative
6	12	A	Healthy	B	>35	>35	32.69	Negative
6	13	A	Healthy	A	40.00	40.00	31.94	Negative
6	13	A	Healthy	B	40.00	40.00	32.45	Negative
6	14	C	Low	A	32.21	32.28	31.49	Suspect
6	14	C	Low	B	32.13	32.3	31.72	Suspect
6	15	F	High	A	28.65	26.08	32.30	Suspect
6	15	F	High	B	29.02	26.89	32.26	Suspect
6	16	D	Medium	A	31.45	29.09	30.95	Suspect
6	16	D	Medium	B	31.41	29.17	31.83	Suspect
6	17	B	Low	A	31.72	29.74	31.54	Suspect
6	17	B	Low	B	31.66	29.69	31.61	Suspect
6	18	E	Medium	A	29.65	29.05	32.46	Suspect
6	18	E	Medium	B	29.54	28.73	32.69	Suspect
6	PAC Cmm	-	-	A	21.00	19.21	40.00	-
6	PAC Cmm	-	-	B	20.57	18.87	40.00	-
6	PAC IAC	-	-	A	40.00	40.00	30.57	-
6	PAC IAC	-	-	B	40.00	40.00	31.12	-
6	NTC	-	-	A	40.00	40.00	40.00	-
6	NTC	-	-	B	40.00	40.00	40.00	-
7	1	C	Low	A	35.12	35.75	28.04	suspect
7	1	C	Low	B	45.00	38.06	28.01	suspect
7	2	C	Low	A	36.16	35.65	28.03	suspect
7	2	C	Low	B	45.00	37.75	28.46	suspect
7	3	E	Medium	A	34.92	34.75	30.00	suspect
7	3	E	Medium	B	34.69	34.20	29.57	suspect
7	4	B	Low	A	31.46	30.95	27.69	suspect
7	4	B	Low	B	31.26	30.92	27.60	suspect
7	5	F	High	A	28.86	28.21	28.11	suspect
7	5	F	High	B	29.35	28.57	28.69	suspect
7	6	E	Medium	A	33.38	34.94	29.28	suspect
7	6	E	Medium	B	33.38	34.20	29.21	suspect
7	7	B	Low	A	32.78	31.99	27.68	suspect
7	7	B	Low	B	32.03	32.36	27.61	suspect
7	8	A	Healthy	A	45.00	45.00	28.72	negative

Lab#	Sample code	Seed lot	Cmm infection level	PCR duplicate	PTSSK (FAM)	MVS21+ (VIC)	Cmt (TexRed)	Cmm validation
7	8	A	Healthy	B	45.00	45.00	28.78	negative
7	9	F	High	A	29.23	28.64	29.29	suspect
7	9	F	High	B	29.84	28.97	29.55	suspect
7	10	D	Medium	A	31.93	31.31	28.20	suspect
7	10	D	Medium	B	32.29	31.54	28.45	suspect
7	11	D	Medium	A	31.98	31.44	28.71	suspect
7	11	D	Medium	B	32.25	31.84	28.87	suspect
7	12	A	Healthy	A	45.00	45.00	28.82	negative
7	12	A	Healthy	B	45.00	45.00	28.63	negative
7	13	A	Healthy	A	45.00	45.00	28.43	negative
7	13	A	Healthy	B	45.00	45.00	28.36	negative
7	14	C	Low	A	37.17	38.36	28.85	suspect
7	14	C	Low	B	37.97	38.42	28.92	suspect
7	15	F	High	A	29.93	29.18	29.44	suspect
7	15	F	High	B	29.76	29.16	29.44	suspect
7	16	D	Medium	A	32.12	31.38	28.22	suspect
7	16	D	Medium	B	31.78	31.38	28.16	suspect
7	17	B	Low	A	32.35	31.98	28.52	suspect
7	17	B	Low	B	33.00	32.13	28.46	suspect
7	18	E	Medium	A	33.79	34.80	29.53	suspect
7	18	E	Medium	B	33.45	34.55	29.74	suspect
7	PAC Cmm	-	-	A	22.99	23.38	45.00	-
7	PAC Cmm	-	-	B	22.97	23.41	45.00	-
7	PAC IAC	-	-	A	45.00	45.00	27.29	-
7	PAC IAC	-	-	B	45.00	45.00	27.23	-
7	NTC	-	-	A	45.00	45.00	45.00	-
7	NTC	-	-	B	45.00	45.00	45.00	-

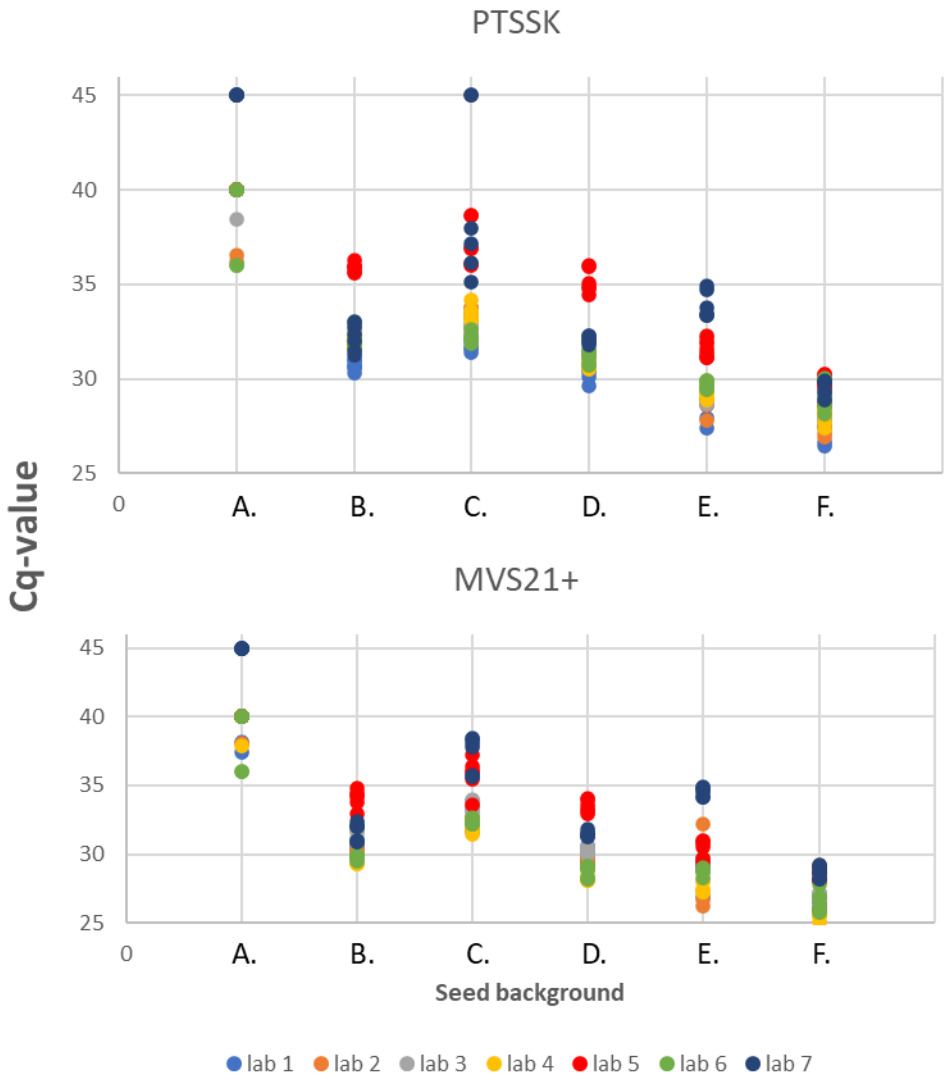

Figure C1. PTSSK and MVS21+ SE-qPCR quantitative results for CT test samples per seed background. Background A is a healthy batch, backgrounds B-F are Cmm infected. Backgrounds B and C are low level infected; D and E are medium level infected and background F is highly infected. Each seed background was processed by seven labs with three subsamples in PCR technical duplicates. All individual datapoints are shown.

Annex D: Clavibacter genomes in Genbank and GT project

Clavibacter%20genomes%20in%20Genbar

Annex E: PTSSK primer and probe alignment with non-Cmm strains

PTSSK%20primer%20and%20probe%20alig