Detection of *Xanthomonas campestris* pv. *campestris* and *Xanthomonas campestris* pv. *raphani* in *Brassica* spp. seed

Addendum Validation report, March 2026

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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

ISF cannot guarantee that laboratories following the protocol 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 tests.

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Detection of *Xanthomonas campestris* pv. *campestris* and *Xanthomonas campestris* pv. *raphani* in *Brassica* spp. seed

SUMMARY

The detection of *Xanthomonas campestris* pv. *campestris* (Xcc) and *Xanthomonas campestris* pv. *raphani* (Xcr) bacteria on untreated *Brassica* seed is assessed by dilution plating on two semi-selective media (mFS and mCS20ABN). A qPCR may be used for identification of the suspect bacterial colonies, which are then confirmed by a pathogenicity assay. A seed extract qPCR assay (SE-qPCR) may be used as a pre-screen (ISF, 2019). A gap analysis performed by the ISHI International Technical Group bean, brassica, pea, and radish showed that the validation of the method (ISHI, 2019) was performed on cabbage seeds only and not on other *Brassica* species. Moreover, validation data for *Xanthomonas campestris* pv. *raphani* (Xcr) was not included for the dilution plating and pathogenicity assays.

The objective of the present study was to perform additional validation experiments and gather data to support the inclusion of Xcr in the dilution plating and pathogenicity assay, and *Brassica* species in the ISHI protocol (2019). The work focussed on three different aspects: 1. generating analytical sensitivity data for detecting Xcc and Xcr with the dilution plating assay (a comparative analysis), 2. generating selectivity data for detecting both Xcc and Xcr with the dilution plating and SE-qPCR assay on *Brassica* species, and 3. generating analytical specificity (inclusivity) data for the detection of Xcr with the pathogenicity assay.

The analytical sensitivity experiment showed that the LOD of the dilution plating assay is between 9-47 CFU/mL seed extract for Xcc and between 13-65 CFU/mL seed extract for Xcr at a 95% confidence level. This meets the requirements set for analytical sensitivity (<100 CFU/mL). Therefore, the dilution plating assay is capable of detecting both Xcc and Xcr with sufficient analytical sensitivity.

The selectivity experiment showed that the dilution plating and SE-qPCR assays generate comparable results for Xcc and Xcr when testing five different *Brassica* species seed lots spiked with Xcc or Xcr dilutions. This demonstrates that the selectivity of both assays is fit for purpose for both species.

The pathogenicity assay performed with 16 different Xcr isolates showed that all isolates cause the typical leaf spot symptoms on a susceptible *Brassica* host and that therefore the inclusivity of the pathogenicity assay for detecting Xcr is fit for purpose.

These results are in support for the inclusion of Xcr and *Brassica* species to the current ISF protocol for the detection of Xcc and Xcr on untreated *Brassica* species seed (ISHI, 2019).

1. INTRODUCTION

Xanthomonas campestris pv. *campestris* (Xcc) and *Xanthomonas campestris* pv. *raphani* (Xcr) are gram-negative, seed-borne bacteria that cause disease on *Brassica* crops. Xcc is the causal agent of black rot, a very important disease of *Brassica* species, and Xcr the causal agent of leaf spot disease of *Brassica* species.

Since 2003, an ISTA/ISHI method has been published for the detection of Xcc/Xcr on untreated *Brassica* seed. In the latest ISHI protocol (version 6.2, October 2019), detection of *Xanthomonas* *campestris* pv. *campestris* (Xcc) and *Xanthomonas campestris* pv. *raphani* (Xcr) bacteria on *Brassica* seed is assessed by dilution plating on two semi-selective media (mFS and mCS20ABN). A qPCR may be used for identification of the suspect bacterial colonies, which are then confirmed by a pathogenicity assay. A seed extract qPCR assay (SE-qPCR) may be used as a pre-screen (ISHI, 2019). Even though the protocol has been in use for many years, the validation of the method was at the time only performed on cabbage seeds (*Brassica oleracea*) and not on other *Brassica* species. Moreover, validation data for *Xanthomonas campestris* pv. *raphani* (Xcr) was not included in any of the validation reports prepared at that time.

The dilution plating assay and pathogenicity assay were evaluated in a comparative test for Xcc in cabbage (*Brassica oleracea*) seed (Koenraadt *et al.*, 2005). An additional study on changed mCS20ABN and FS media recipes for Xcc on cabbage was performed by Sato *et al*. (2014). The identification qPCR was validated by Barnhoorn (2018) for Xcc and Xcr and the SE-qPCR by Bruinsma *et al*. (2018) for Xcc in cabbage seed.

To support the inclusion of Xcr and broader *Brassica* species in the protocol, the ISHI International Technical Group for bean, brassica, pea, and radish agreed to carry out additional validation studies. To justify the inclusion of Xcr in the protocol, an experiment to demonstrate equivalent sensitivity of the dilution plating assay for both Xcc and Xcr, and an inclusivity experiment to confirm the Xcr detection in the pathogenicity assay were performed. For the qPCR assay, relevant data on Xcr was already available in the Barnhoorn (2018) report.

To extend the protocol beyond *Brassica oleracea*, selectivity experiments were performed using both the dilution plating and SE-qPCR assays, demonstrating that both Xcc and Xcr, at a concentration close to the LOD, can be detected in multiple *Brassica* species. Since the identification qPCR and pathogenicity assay are performed using pure cultures, no additional selectivity testing was deemed necessary for those assays.

2. OBJECTIVES

The objective of this study was to perform additional validation experiments to gather data in support of the inclusion of Xcr in the dilution plating and pathogenicity assay and *Brassica* species to the protocol.

The present study includes:

- Analytical sensitivity experiment for the validation of the dilution plating assay for both Xcc and Xcr,

- Selectivity experiment for the validation of the dilution plating assay and SE-qPCR for both Xcc and Xcr,

- Analytical specificity (inclusivity) experiment for the validation of the pathogenicity assay for Xcr.

The additional validation was performed by Bejo Zaden in the period July-October 2024. The validation was performed according to the ISHI guidelines for the Validation of Seed Health Methods (ISHI, 2020).

3. METHOD VALIDATION

For the different validation experiments, the seed lots and bacterial cultures described in Table 1 and 2, respectively, were used.

Table 1. Seed lots used in the validation experiments.

Code	Crop species	Scientific name
A	White cabbage	*B. oleracea* convar. *capitata* var. *alba*
B	Borecole	*B. oleracea* convar. *acephala* var. *laciniata*
C	Pak choy	*B. rapa* subsp. *chinensis*
D	Rutabaga	*B. napus* subsp. *rapifera (napobrassica)*
E	Turnip	*B. rapa* subsp. *rapa*

Table 2. Bacterial isolates used in the validation experiments.

Code	Species	Origin			
		Crop species	Leaf/Seed	Country	Year
P4123	Xcc	White cabbage	Seed	US	2006
P4077	Xcr	Cabbage	Leaf	Germany	2006
55	Xcr	Red cabbage	Leaf	Italy	2017
67	Xcr	Kohlrabi	Leaf	Australia	2021
76	Xcr	Red cabbage	Leaf	Australia	2021
85	Xcr	Broccoli	Leaf	US	2022
87	Xcr	Cauliflower	Leaf	Australia	2022
89	Xcr	Kohlrabi	Leaf	Australia	2022
NBC-269 (XCA 2)	Xcr	Cabbage	Seed	US	2003
53373-17	Xcr	Cabbage	Seed	US	2009
127/5/071	Xcr	Cabbage	Seed	US	2009
134/12/3220	Xcr	Cabbage	Seed	US	2009
NBC-702	Xcr	Broccoli	Leaf	Netherlands	2006
NBC-4172	Xcr	Kohlrabi	Seed	Czech Republic	2012
NBC-5257	Xcr	Radish	Seed	Italy	2016
NBC-5484	Xcr	Cabbage	Leaf	Netherlands	2016
NBC-5723	Xcr	Radish	Leaf	Marocco	2018

3.1. Analytical sensitivity dilution plating assay

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

To justify the inclusion of Xcr in the protocol, an experiment to demonstrate equivalence in the sensitivity of the dilution plating assay for both Xcc and Xcr was performed. The analytical sensitivity requirements will be met when the limit of detection (LOD), the lowest concentration of bacteria at which Xcc and Xcr is consistently detected, is minimally 100 CFU/mL with 95% confidence.

Experimental approach

Analytical sensitivity of the dilution plating assay was determined by spiking healthy seed extract samples with a dilution series of liquid cultured Xcc (P4123) and Xcr (P4077). To check for possible inhibition in the seed extract background on the recovery of the bacteria, the dilution series was also spiked into the buffer without seed extract.

Before the start of the actual validation experiment, the most suitable dilutions were established based on experimental results (around the expected LOD and above; data not shown).

Preparation of the dilution series to determine Xcc and Xcr concentration

A single growing culture for both isolates was prepared by inoculating 1 µL of 3-4 days old YDC culture in a flask with 50 mL sterile liquid nutrient broth. The flasks were swirled at 150 rpm at 28 °C. After 24 hours, the cultures were used to prepare three independent five-fold dilution series (named V1-V2-V3) of five steps each (named V1.1-V1.5, V2.1-V2.5 etc.) in sterile 0.15 M sodium chlorine (NaCl) without Tween^{TM} 20.

The three five-fold dilution series (V1-V2-V3) were plated in triplicate (100 µL/plate) on mCS20ABN medium to determine the Xcc and Xcr concentrations. Earlier results (data not shown) suggested that mCS20ABN medium is the most informative medium and gives the best recovery. Plates were incubated at 28 °C and scored after three days.

Spiking of the dilution series

Seed lot A (Table 1) was used to prepare a seed extract. A bulk seed extract (SE) was prepared by shaking five subsamples of 10,000 seeds in 100 mL of 0.15 M sterile NaCl with Tween 20 (NaCl+T), according to the ISHI protocol (Annex A). After an incubation of 2.5 hours with shaking at 125 rpm at room temperature (20–25 °C), the seed extracts of the subsamples were collected in a sterile flask.

A volume of 1 mL of the three independent five-fold dilution series was spiked into 10 mL of SE and NaCl+T giving rise to 15 spiked SE and NaCl+T samples each. In parallel and as a negative control, 1 mL of NaCl was spiked in triplicate into 10 mL of SE and NaCl+T (36 samples total). After spiking, a sample of 1 mL spiked SE and NaCl+T samples, together with the negative controls, were used for dilution plating on mCS20ABN (3×100 µL) and mFS (3×100 µL), according to the ISHI protocol (Annex A).

The analytical sensitivity of the dilution plating assay corresponds to the lowest concentration in which Xcc or Xcr was detected in all three dilution series on one or both semi-selective media (mCS20ABN and mFS).

Results

Xcc and Xcr concentrations of the dilution series

The raw dilution plating results for Xcc and Xcr for each concentration of the dilution series for mCS20ABN are presented in Annex B.

The number of CFU of each pathogen per dilution was calculated based on the average CFU of dilution 3 (1:125) after dilution plating on mCS20ABN medium (Annex B). Dilution 3 was used as this was the first dilution to always give single colonies that could be enumerated (not crowded or overlapping colonies).

For Xcc the calculations are as follows: with the average CFU of 51.8 CFU/100 µL, 518 CFU (1 mL) is added to 10 mL SE or NaCl+T (final volume was at 11 mL), leading to an expected CFU of 47.1

CFU/mL or 4.71 CFU/100 µL for dilution 3. For the other dilutions this number was calculated by dividing or multiplying by five (the dilution factor between each dilution).

For Xcr, the calculations are as follows: 712 CFU (1 mL) is added to 10 mL SE or NaCl-T, leading to an expected 64.7 CFU/mL or 6.47 CFU/100 µL for dilution 3. For the other dilutions this number was again calculated by dividing or multiplying by five (the dilution factor between each dilution).

Dilution plating test samples

The raw plating results for the analytical sensitivity are presented in Annex C for mCS20ABN and Annex D for mFS. The Xcc and Xcr positive plate counts on mCS20ABN and mFS media, per dilution, spiked in SE and NaCl+T are shown in Table 3.

Table 3. Number of positive results of the Xcc and Xcr dilutions spiked in SE and NaCl+T on mCS20ABN and mFS agar plates.

Target	Dilution	Calculated CFU/100 µL[a]	mCS20ABN		mFS	
			Seed extract	NaCl+T	Seed extract	NaCl+T
Xcc	1	117.7	9+/9	9+/9	9+/9	9+/9
	2	23.5	9+/9	9+/9	9+/9	9+/9
	3	4.7	9+/9	9+/9	9+/9	9+/9
	4	0.9	7+/9	5+/9	7+/9	6+/9
	5	0.2	0+/9	1+/9	3+/9	0+/9
Xcr	1	161.8	9+/9	9+/9	9+/9	9+/9
	2	32.4	9+/9	9+/9	9+/9	9+/9
	3	6.5	9+/9	9+/9	9+/9	9+/9
	4	1.3	6+/9	9+/9	8+/9	5+/9
	5	0.3	4+/9	3+/9	2+/9	3+/9

[a] Calculation based on the average colony count of dilution 3 plated on mCS20ABN directly (see Annex B), 1 mL dilution spiked to 10 mL SE or NaCl+T, of which 100 µL is plated.

No inhibition by the SE background was observed as there is no significant difference between the average plating results, including standard deviations, of the spiked dilution series in SE and NaCl-T (Annex C and D). All samples spiked with NaCl (negative control) were plating negative (no colonies detected; Annex C and D). All samples with SE from Lot A spiked with Xcc or Xcr dilutions 1, 2, 3 generated dilution plating positive results on all three replicates on both semi-selective media (CS20ABN or mFS; Table 3).

The samples with SE from Lot A spiked with Xcc or Xcr dilution 4 generated dilution plating positive results for all three dilution series in at least one of the three repetitions on at least one of the semi-selective media but not in all replications.

Dilution 5 was detected in less than half of the spiked SE. Therefore, the LOD for both media and both Xcc and Xcr is in between dilution 3 and dilution 4. The calculated CFU/mL of dilution 4, based on the average colony count of dilution 3 plated on mCS20ABN directly, is 9 CFU/mL for Xcc, and 13 CFU/mL for Xcr. The calculated CFU/mL of dilution 3, based on the average colony count of dilution 3 plated on mCS20ABN directly is 47 CFU/mL for Xcc, and 65 CFU/mL for Xcr.

Conclusion

The LOD of the Xcc and Xcr dilution plating assay at a 95% confidence level is between 9 and 47 CFU/mL SE for Xcc and between 13 and 65 CFU/mL SE for Xcr on both mCS20ABN and mFS media. This meets the requirements set for analytical sensitivity requirements (<100 CFU/mL). There is little difference between the analytical sensitivity for Xcc and Xcr in the dilution plating assay, allowing for the consistent detection of both pathogens.

3.2. Selectivity dilution plating assay and SE-qPCR

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

To extend the dilution plating and qPCR protocols beyond *Brassica oleracea*, selectivity experiments were performed to demonstrate that both Xcc and Xcr can be detected in multiple *Brassica* species.

The selectivity requirements for dilution plating and SE-qPCR will be met if the assay can detect Xcc and Xcr bacteria at a concentration close to the LOD, as determined in Section 3.1, leading to a 100% detection in different *Brassica* species matrices.

Experimental approach

Selectivity of the dilution plating assay and SE-qPCR was determined by spiking healthy seed extract samples from five different *Brassica* species (Table 1) with liquid cultured Xcc (P4123) and Xcr (P4077) at a concentration close to the LOD. To check for possible inhibition in the seed extract background on the recovery of the bacteria, the liquid cultured bacteria were also spiked into the buffer without seed extract.

A growing culture for both isolates was prepared by inoculating 1 µL of a 3-4 day-old YDC culture in a flask with 50 mL sterile liquid nutrient broth. The flasks were swirled at 150 rpm at 28 °C. After 24 hours, the cultures were used to prepare one five-fold dilution series (V1.1-V1.4) in sterile 0.15 M NaCl+T. The five-fold dilution series (V1.1-V1.4) was plated in triplicate (100 µL/plate) on mCS20ABN and Kings B medium (non-selective medium) to determine the Xcc and Xcr concentrations. Plates were incubated at 28 °C and scored after three days.

Five seed lots, named lot A-E (Table 1), were used to prepare seed extracts. A seed extract (SE) from each lot was prepared by incubating one subsample of 10,000 seeds in 100 mL of 0.15 M sterile NaCl+T, according to the ISHI protocol (Annex A). After an incubation of 2.5 hours with shaking at 125 rpm at room temperature (20 – 25 °C), the seed extracts of the subsamples were collected in a sterile flask.

A volume of 20 mL of each SE and NaCl+T was spiked with 2 mL of NaCl or with 2 mL of the dilution from the Xcc and Xcr dilution series just above the LOD. Before the start of the experiment, the most suitable dilution just above the LOD were established based on the results of the analytical sensitivity experiment.

After spiking, a sample of 2 mL spiked SE or NaCl+T was used for dilution plating on CS20ABN (3×100 µL) and mFS (3×100 µL), according to the ISHI protocol (Annex A).

The remaining 20 mL spiked SE or NaCl+T was analysed by SE-qPCR. A volume of 10 mL SE or NaCl+T with Xcc or Xcr was spiked with 5 µL of *Acidovorax cattleyae* (Acat), as the internal amplification control, resulting in a Cq value around 28 (based on earlier experiments, data not shown).

For the centrifugation steps, the ISHI protocol was used. For the DNA extraction the Roche MP96 extraction platform was used. The qPCR reaction mix (FastStart Essential DNA Probes Master from Roche, Basel, Switzerland) and cycling conditions were used according to the Bejo protocol, using the same qPCR assays as the ISHI method. In earlier experiments, it was shown that results with the Bejo protocol (Roche MP96 and Roche PCR mix) are equivalent with the ISHI protocol (Bejo internal data). Each qPCR reaction was performed in triplicate.

Results

Xcc and Xcr concentration of the dilution series

The plating results for Xcc and Xcr for each concentration of the dilution series for mCS20ABN and KB are presented in Table 5. Results confirmed a linear five-fold concentration difference between dilutions, and similar average plating results between the selective (mCS20ABN) and non-selective (KB) media. Based on these results, dilution 3 (1:125) was spiked to the seed extracts for Xcc, and dilution 4 (1:625) for Xcr.

Table 5. Results of CFU/100 µL of the dilution series for Xcc and Xcr in preparation for the analytical selectivity evaluation. N/A: Not applicable, Rep: Repetition, SD: Standard deviation, TNCT: Too numerous to count (>300 colonies).

	Dilution	**CFU/100 µL of Xcc**				**CFU/100 µL of Xcr**			
		Rep 1	**Rep 2**	**Rep 3**	**Average (SD)**	**Rep 1**	**Rep 2**	**Rep 3**	**Average (SD)**
mCS20AB	1	TNTC	TNTC	TNTC	N/A	TNTC	TNTC	TNTC	N/A
	2	TNTC	TNTC	TNTC	N/A	TNTC	TNTC	TNTC	N/A
	3	71	62	57	63.3 (±5.8)	TNTC	TNTC	TNTC	N/A
	4	11	5	17	11.0 (±4.9)	91	72	58	73.7 (±13.5)
KB	1	TNTC	TNTC	TNTC	N/A	TNTC	TNTC	TNTC	N/A
	2	TNTC	TNTC	TNTC	N/A	TNTC	TNTC	TNTC	N/A
	3	45	78	60	61.0 (±13.5)	TNTC	TNTC	TNTC	N/A
	4	7	11	15	11.0 (±3.3)	76	81	77	78.0 (±2.2)

Dilution plating test samples

The raw plating results for the selectivity are listed in Annex E for Xcc and Annex F for Xcr. The Xcc and Xcr average colony counts on mCS20ABN and mFS media, spiked in the five seed lots (coded A-E) and NaCl+T are shown in Table 6. All samples spiked with NaCl+T (negative control) were plating negative (no colonies; Annex E and F). All samples with SE from seed lots A-E spiked with Xcc or Xcr dilutions just above the LOD gave positive results by dilution plating for all three replicates on both semi-selective media CS20ABN/mFS (Annex E and F). No inhibition by the SE background was observed as no significant difference between the average plating results, including standard deviation (SD), of the spiked dilution series in SE and NaCl+T was recorded (Table 6).

SE-qPCR test samples

The raw SE-qPCR results for the controls for the selectivity are listed in Annex G. Results for the qPCR control samples were as expected (PACs were positive; NTC (sterile PCR-grade water) negative).The raw SE-qPCR results for NaCl+T spiked samples for the selectivity are listed in Annex H and for Xcc dilution 3 (1:125) and Xcr dilution 4 (1:625) spiked samples in Annex I. Qualitative results are summarised in Table 7. Average Cq results for Xcc and Xcr, spiked in the five seed lots and in NaCl+T are shown in Figure 1.

Table 6. Average plating results in CFU/100 µL with standard deviations of the Xcc dilution 3 (1:125) and Xcr dilution 4 (1:625) spiked in SE from seed lots A-E and NaCl+T on mCS20ABN and mFS agar.

Target	Lot	CFU on mCS20ABN	CFU on mFS
Xcc	**NaCl+T**	5.3 (±3.2)	7.0 (±3.6)
	A	3.0 (±2.0)	4.7 (±2.5)
	B	9.7 (±1.5)	6.3 (±2.3)
	C	9.0 (±2.6)	6.0 (±3.6)
	D	5.3 (±1.2)	6.7 (±3.2)
	E	4.0 (±1.0)	1.3 (±0.6)
	Average Lot A - E	6.2 (±3.0)	5.0 (±2.9)
Xcr	**NaCl+T**	5.0 (±4.6)	7.7 (±0.6)
	A	5.3 (±0.6)	6.7 (±0.6)
	B	7.7 (±1.5)	10.0 (±3.6)
	C	6.3 (±1.2)	5.0 (±2.0)
	D	5.7 (±2.1)	8.0 (±3.5)
	E	6.0 (±1.7)	7.0 (±1.0)
	Average Lot A - E	6.2 (±1.5)	7.3 (±2.6)

Table 7. Number of positive results (Cq<35) for the seed lots and NaCl+T spiked with NaCl, Xcc dilution 3 (1:125) and Xcr dilution 4 (1:625).

Lot	NaCl			Xcc			Xcr		
	Kohl	Berg	Acat	Kohl	Berg	Acat	Kohl	Berg	Acat
NaCl+T	0+/6	0+/6	6+/6	2+/3	3+/3	3+/3	0+/3	0+/3	3+/3
A	0+/6	2+/6	6+/6	3+/3	3+/3	3+/3	0+/3	3+/3	3+/3
B	1+/6	2+/6	6+/6	3+/3	3+/3	3+/3	0+/3	3+/3	3+/3
C	0+/6	0+/6	6+/6	3+/3	3+/3	3+/3	0+/3	3+/3	3+/3
D	0+/6	0+/6	6+/6	3+/3	3+/3	3+/3	0+/3	1+/3	3+/3
E	6+/6	6+/6	6+/6	3+/3	3+/3	3+/3	1+/3	3+/3	3+/3

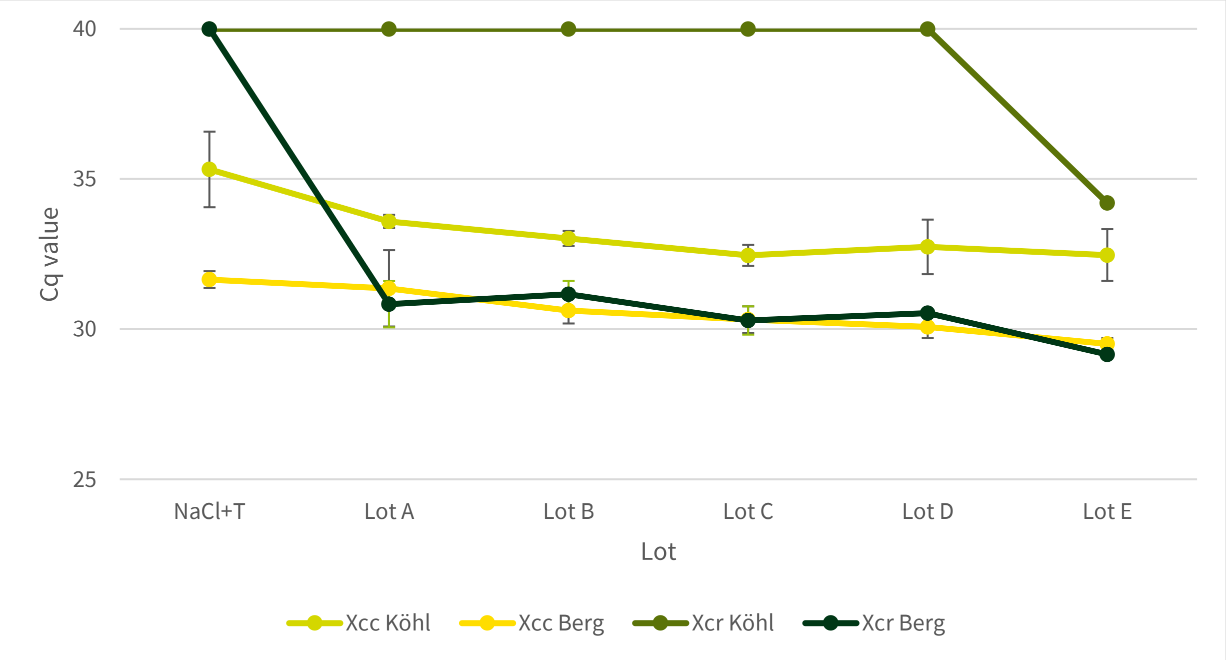

Figure 1. Average Cq values of Xcc and Xcr spiked SE from seed lots A – E and NaCl+T. Error bars represent the standard deviation. Cq 40 represents no amplification detected.

The internal amplification control (Acat) was detected in all the replicates of the NaCl and Xcc/Xcr spiked samples (Table 7) with comparable Cq values (average Cq 27.17 ± 0.55 and Cq 27.19 ± 0.77, respectively; Annexes H and I). No amplification of Xcc and Xcr was detected in the NaCl+T control samples spiked with NaCl (negative process control; Table 7). In the seed lots A, B and E spiked with NaCl (negative control), some positive signals (Cq<35) were detected for the Berg and/or Kohl primers.

Spiking with Xcc resulted in a positive signal in all replicates for both the Kohl and Berg assays for seed lots A-E, with comparable Cq values (Figure 1). However, for NaCl+T spiked with Xcc, one of the replicates had a Cq value above the qPCR cut-off (Table 2), which resulted in an average Cq above the Cq cut-off of 35 (Figure 1).

Spiking with Xcr resulted in a positive signal in all replicates for the Berg assay for seed lots A-C and E. However, in seed lot D, two out of three replicates showed no amplification, while the third replicate had a clear signal (Cq = 30.54). For NaCl+T spiked with Xcr, no amplification was observed. Furthermore, one out of three replicates for the Kohl assay for Xcr spiked in seed lot E was found positive.

Overall, the average Cq results of Xcc and Xcr spiked SE from seed lots A-E show comparable Cq values (Figure 1).

Conclusion

Although amplification was expected for the negative Xcr spiked replicates for NaCl+T and seed lot D, this lack of amplification does not affect the selectivity evaluation. Furthermore, the positive signals (Cq<35) detected for the Berg and/or Kohl primers in the seed lots A, B and E spiked with NaCl (negative control) are an indication of a DNA contamination of Xcr for seed lot A and Xcc for seed lot B and E, as dilution plating results were negative (Annex E). The one positive replicate for the Kohl assay for Xcr spiked in seed lot E can be related back to the positive results seen for seed lot E when spiking with NaCl.

The dilution plating and SE-qPCR results were all positive for the different seed lots spiked with Xcc or Xcr at a concentration around the LOD. This meets the requirements set for analytical selectivity of both assays.

3.3. Analytical specificity pathogenicity assay for Xcr

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

Previous work has already shown the exclusivity capacity of the pathogenicity assay (Koenraadt *et al.*, 2005). As such, the experiments described here focussed on determining the inclusivity of the pathogenicity assay for Xcr to justify the inclusion of Xcr in the protocol. Therefore, the specificity requirements for the pathogenicity assay for Xcr will be met if typical Xcr disease symptoms will develop in the assay with target isolates.

Experimental approach

In order to validate the inclusivity of the pathogenicity assay for Xcr, sixteen available Xcr isolates from different geographical origins (at least eight countries), years (nine years) and crop types were used (six crops; Table 2). Each isolate and a mock control was stabbed in two secondary leaf veins of one leaf of *Brassica* variety Golfer, according to the protocol in Annex A, and assessed one and two weeks later.

<u>Results</u>

All sixteen isolates cause the typical Xcr leaf spot symptoms on the leaf. Examples of symptom expression for seven isolates is shown in Figure 2.

<u>Conclusion</u>

The pathogenicity assay produces typical Xcr disease symptoms with the sixteen Xcr isolates tested. This meets the requirements set for analytical specificity for the pathogenicity assay for Xcr.

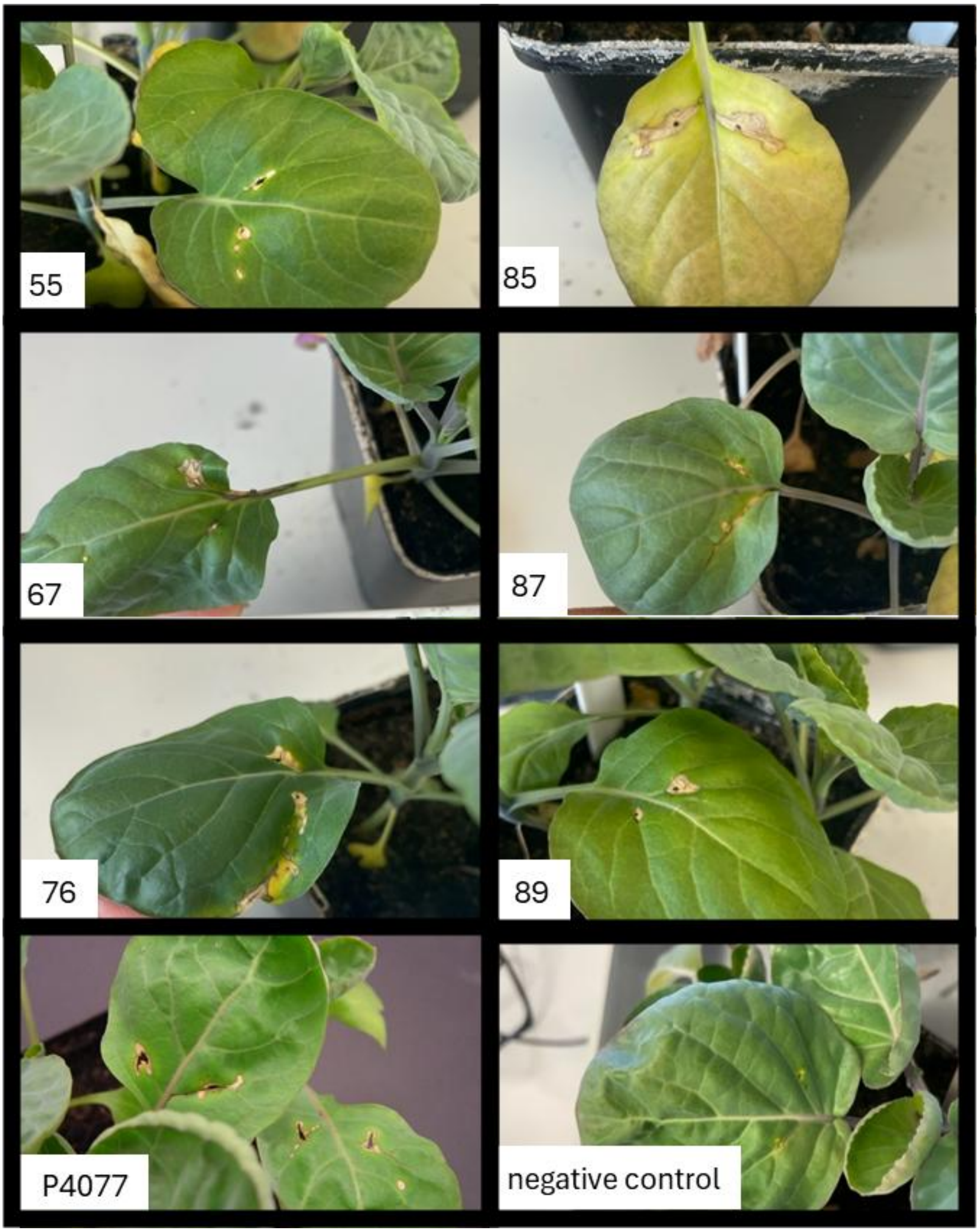

Figure 2. Examples of leaf spot symptoms caused by seven Xcr strains (55, 67, 76, 85, 87, 98, P4077 and negative control) two weeks after inoculation.

4. CONCLUSION

The sensitivity requirements for dilution plating assay are met since there is no difference observed between the analytical sensitivity for Xcc and Xcr.

The selectivity requirements for dilution plating and SE-qPCR assays are met since both assays can detect Xcc and Xcr bacteria at a concentration close to the LOD in different *Brassica* species matrices.

The specificity requirements for the inclusivity of the pathogenicity assay for Xcr bacteria are met since all strains tested could be detected.

These results are in support for the inclusion of both Xcr and *Brassica* species to the current ISHI protocol.

5. REFERENCES

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6. ANNEXES

Annex A. Protocol for detecting *Xanthomonas campestris* pv. *campestris* and pv. *raphani* on *Brassica* spp. seed

SAMPLE AND SUBSAMPLE SIZE

The recommended minimum sample size is 30,000 seeds with a maximum subsamples size of 10,000 seeds.

I. PRE-SCREEN BY SEED-EXTRACT qPCR (OPTIONAL STEP)

Materials

- Conical flasks or equivalent
- Orbital shaker
- 0.85% (w/v) NaCl with 0.02% (v/v) Tween™ 20 added after autoclaving
- qPCR mix, primers (Table I.1) and PCR equipment
- Controls (Table I.2)
- DNA isolation kit e.g., Qiagen DNeasy Blood and Tissue kit
- Centrifuge

Table I.1. Primer-sequences and references.

Name	Target	Sequence (5’– 3’)	Source
XCC-F	Xcc	GTG CAT AGG CCA CGA TGT TG	Köhl *et al.*, 2011
XCC-R		CGG ATG CAG AGC GTC TTA CA	
XCC-Pr		FAM – CAA GCG ATG TAC TGC GGC CGT G – NFQ-MGB	
DLH153-F	Xcc	GTA ATT GAT ACC GCA CTG CAA	Berg *et al.*, 2006
DLH154-R		CAC CGC TCC AGC CAT ATT	
P7		VICrepl – ATG CCG GCG AGT TTC CAC G – BHQ1	
Acat2-F	Acat	TGT AGC GAT CCT TCA CAA G	Koenraadt *et al.*, 2014
Acat2-R		TGT CGA TAG ATG CTC ACA AT	
Acat1-Pr		Texas Red – CTT GCT CTG CTT CTC TAT CAC G – BHQ2	
Contig21-F	Acit	ACC GAA CAG AGA GTA ATT CTC AAA GAC	Sudarshana, 2010
Contig21-R		GAG CGT GAT GGC CAA TGC	
Contig21-Pr		FAM – CAT CGC TTG AGC AGC AA – 3IABkFQ	

Table I.2. Types of controls used.

Control type	**Description**
Positive extraction control (PEC)[a]	*A. cattleyae* spike *or*
	A. citrulli spike
Negative process control (NPC)	Seed extract from healthy seeds or buffer
Non template control (NTC)	PCR reaction mix with water
Positive amplification control (PAC)	Xcc DNA *and*
	Xcr DNA
Positive process control (PPC)	Positive seed sample

[a] The PEC also serves as internal amplification control (IAC).

1. Seed extraction

1.1. Suspend each subsample of seeds, and the positive process control (PPC), separately, in pre-chilled (2–4 °C) sterile 0.85% (w/v) NaCl with 0.02% (v/v) Tween™ 20 in a conical flask or equivalent. The volume of NaCl should be adjusted according to the number of seeds used (use 10 mL of buffer for 1,000 seeds).

1.2. Shake on an orbital shaker at 100–125 rpm for 2.5 hours at room temperature (20–25 °C).

1.3. Shake flasks to mix prior to further processing.

2. Spiking samples

2.1. Transfer 10 mL of seed extract from each subsample into a 15 mL centrifuge tube. Include a seed extract from healthy seeds or buffer without seeds as a negative process control (NPC) (Table I.2).

2.2. Add a positive extraction control (PEC) spike to all subsamples, the PPC and NPC for seed extract qPCR (e.g., 100 µL of *A. cattleyae* **or** *A. citrulli* stock of OD_{600} = 0.6) (Table I.2).

3. Concentration of bacteria

3.1. Centrifuge sample tubes at 1,200 × *g* for 5 min at room temperature. Carefully transfer the supernatant to a fresh 15 mL tube and discard the pellet.

3.2. Centrifuge the supernatant at a minimum of 3,400 × *g* for 20 min at room temperature.

3.3. Carefully decant as much supernatant as possible without disturbing the pellet.

3.4. Use the pellet for the isolation of DNA.

Note: The pellet is not always stable. Take tubes carefully out of the centrifuge.

4. DNA isolation

4.1. Re-suspend the bacterial pellet in lysis buffer and extract the bacterial DNA, as described in the manual of the DNA extraction kit.

Note: The assay has been validated with the Qiagen DNeasy Blood and Tissue kit. If a different DNA isolation kit is used, it is necessary to verify its performance.

5. qPCR

Notes: Work on ice whenever possible and minimize probe's exposure to light.

If different qPCR mixtures and amplification programs are used, it is necessary to verify their performance.

5.1. Use the Xcc primer sets from Köhl *et al*. (2011) and from Berg *et al*. (2006) (Table I.1).

5.2. Include the appropriate primers and probes for the spike used (Section 2). To detect the spike *A. cattleyae,* use the primer set from Koenraadt *et al*. (2014). To detect the spike *A. citrulli*, use the primer set from Sudarshana (2010) (Table I.1).

5.3. Prepare the qPCR mix (Table I.3).

5.4. Test each DNA extraction from each subsample in duplicate (technical replicates). Include both positive amplification controls (PAC) and a non-template control (NTC) (Table I.2).

5.5. Cover the plate after adding DNA and perform the qPCR according to the PCR conditions (Table I.4).

Table I.3. qPCR mix for the SE-qPCR[a].

Component	Per reaction (in µL)	Final concentration
PCR grade water	9.95	
PerfeCTa Multiplex qPCR ToughMix (5×)	5.00	1×
XCC-F (10 µM)	0.75	0.30 µM
XCC-R (10 µM)	0.75	0.30 µM
XCC-Pr (10 µM)	0.50	0.20 µM
DLH153-F (10 µM)	0.75	0.30 µM
DLH154-R (10 µM)	0.75	0.30 µM
P7 (10 µM)	0.50	0.20 µM
Acat2-F or contig21-F (10 µM)	0.40	0.16 µM
Acat2-R or contig21-R (10 µM)	0.40	0.16 µM
Acat1-Pr or contig21-Pr (10 µM)	0.25	0.10 µM
Sample	5.00	
Total	**25.00**	

[a] Use of a triplex qPCR, as described in this method, is preferred. If, however, the PCR machine used is not appropriate for a triplex, two duplex PCR reactions (target and spike) can be performed, which however will need to be internally validated.

Table I.4. PCR conditions SE-qPCR.

Step	Temperature	Duration
hold	95 °C	2 min
40 cycles	95 °C	15 sec
	60 °C	48 sec

6. Interpretation and decisions

Cut-off values must be established by each laboratory for their positive and internal amplification controls prior to the assay being used on routine samples. For recommendations on setting cut-off values, see Real-time PCR, an 'indirect' test used for pre-screening in seed health methods. The cut-off for *A. cattleyae* is set preferably between Cq 28 and 32, and for *A. citrulli* below 35.

A cut-off value of Cq 35 for the SE-qPCR of samples was used for the validation study.

For interpretation and decision making, results from both primer sets need to be considered, see Table I.5. Test results are only valid when all included controls presented in Table I.2 give the expected results.

Table I.5. Interpretation and decision table SE-qPCR.

Köhl qPCR	Berg qPCR	qPCR result	Follow-up
Positive	Positive	Target DNA for Xcc detected	Dilution plating
Negative	Positive	Target DNA for Xcr detected	Dilution plating
Positive	Negative	Inconclusive	Dilution plating
Negative	Negative	No target DNA for Xcc/Xcr detected	No follow up required

II. DETECTION BY DILUTION PLATING

Materials

- Dilution bottles
- Incubator: operating at 28–30 °C
- NaCl (0.15 M) (Table II.1)
- pH meter
- Sterile spreader
- Controls (Table II.2)
- Pipettes and tips
- Plates of mFS, mCS20ABN and YDC media (Tables II.3-II.5)

Table II.1. NaCl 0.15M[a].

Compound	Amount/L
Sodium chloride (NaCl)	8.5 g

[a] For extraction of seeds, add 20 µL of sterile Tween™ 20 per 100 mL after autoclaving.

Table II.2. Types of controls used.

Control type	Description
Positive control (PC)	A known strain of Xcc *and*
	A known strain of Xcr
Negative process control (NPC)	Extraction buffer (NaCl with Tween™ 20)

Table II.3. mFS semi-selective medium (Schaad *et al.*, 1989).

Compound	Amount/L
K_2HPO_4	0.8 g
KH_2PO_4	0.8 g
KNO_3	0.5 g
$MgSO_4.7H_2O$	0.1 g
Yeast extract	0.1 g
Methyl Green (1% (w/v) aqueous solution)	1.5 mL
Soluble starch (Merck 1252)	25.0 g
Agar (BD Bacto™ Agar)	15.0 g
Nystatin[a] (10 mg/mL in 50% (v/v) ethanol)	35 mg (3.5 mL)
D-Methionine[a] (1 mg/mL in 50% (v/v) ethanol)	3 mg (3.0 mL)
Pyridoxine HCl[a] (1 mg/mL in 50% (v/v) ethanol)	1 mg (1 mL)
Cephalexin[a] (20 mg/mL in 50% (v/v) ethanol)	50 mg (2.5 mL)
Trimethoprim[a] (10 mg/mL in 70% (v/v) ethanol)	30 mg (3 mL)

[a] Added after autoclaving.

Table II.4. mCS20ABN semi-selective medium.

Compound	Amount/L
Soya peptone	2.0 g
Tryptone (BD Bacto™ Tryptone)	2.0 g
KH_2PO_4	2.8 g
$(NH_4)_2HPO_4$	0.8 g
$MgSO_4.7H_2O$	0.4 g
L-Glutamine	6.0 g
L-Histidine	1.0 g
D-Glucose (dextrose)	1.0 g
Soluble starch (Merck 1252)	25.0 g
Agar (BD Bacto™ Agar)	18.0 g
Nystatin[a] (10 mg/mL in 50% (v/v) ethanol)	35 mg (3.5 mL)
Neomycin sulphate[a] (20 mg/mL in distilled/deionised water)	40 mg (2.0 mL)
Bacitracin[a] (50 mg/mL in 50% (v/v) ethanol)	100 mg (2.0 mL)

[a] Added after autoclaving.

Media preparation

1. Weigh all ingredients, except the antibiotics, pyridoxine-HCl and D-methionine.

Note: For starch hydrolysis the source of starch used in the selective media is critical. Verify that each new batch of starch gives clear zones of hydrolysis with reference cultures of Xcc.

2. Dissolve them in a suitable container in distilled/deionised water.
3. Adjust pH to 6.5, if necessary.
4. Autoclave at 15 psi for 15 min at 121 °C.
5. Prepare antibiotics, pyridoxine-HCl and D-methionine solutions, and filter sterilize as appropriate.

Note: Antibiotics stock solutions and other supplements prepared in distilled/deionised water must be sterilized using a 0.2 µm bacterial filter. Alternatively, add the antibiotic powder to the autoclaved distilled/deionised water. Solutions prepared in 70% (v/v) ethanol do not need sterilization.

6. Allow medium to cool to approximately 50 °C prior to adding antibiotics, pyridoxine-HCl and D-methionine solutions.
7. Mix the molten medium thoroughly but gently to avoid air bubbles and pour plates (18 mL per 90 mm plate).
8. Leave plates to cool down and dry under sterile conditions.

Note: Storage conditions and duration may affect antibiotic activity, which can influence the performance of the test. Depending on the source of starch, pre-storage of plates in the refrigerator (4 °C) for at least four days before use may result in more easily visible zones of starch hydrolysis.

Table II.5. Yeast dextrose chalk (YDC) agar (Wilson *et al.,* 1967).

Compound	Amount/L
Agar (BD Bacto™ Agar)	15.0 g
Yeast extract	10.0 g
$CaCO_3$ (light powder)	20.0 g
D-Glucose (dextrose)	20.0 g

Preparation

1. Weigh all ingredients and put them into a suitable oversized container (e.g., 250 mL of medium in a 500 mL bottle/flask) to allow swirling of the medium just before pouring.
2. Dissolve in 1 L of distilled/deionised water by steaming the mix.
3. Autoclave at 15 psi for 15 min at 121 °C and allow the medium to cool to approximately 50 °C.
4. Swirl the bottle/flask to ensure an even distribution of $CaCO_3$ and avoid air bubbles. Pour 22 mL on to each 90 mm plate.
5. Leave plates to cool down and dry under sterile conditions.

1. Dilution and plating

1.1. Perform the seed extraction as described in section I.1.

1.2. Prepare two serial ten-fold dilutions from the seed extract by i.) Pipetting 0.5 mL of the extract into 4.5 mL of sterile NaCl to give a 10^{-1} dilution and ii.) Pipetting 0.5 mL of the 10^{-1} dilution into 4.5 mL of sterile NaCl to give a 10^{-2} dilution. Vortex all dilutions well.

1.3. Pipette 100 µL of each dilution and the undiluted seed extract onto plates of the mFS and mCS20ABN selective media and spread over the surface.

1.4. Incubate plates at 28–30 °C and examine after 3–4 days.

2. Positive control (PC) - culture or reference material

2.1. Prepare a suspension of a known Xcc and Xcr strain in sterile NaCl or reconstitute standardized reference material according to the supplier's instructions.

Note: Xcc and Xcr cannot be distinguished by their colony morphology.

2.2. Dilute sufficiently to obtain dilutions containing approximately 10^2 to 10^4 CFU/mL.

2.3. Pipette 100 µL of appropriate dilutions onto plates of both semi-selective media (mFS, mCS20ABN) and spread over the surface.

2.4. Incubate plates with the sample plates (as in Section 1.4).

3. Negative process control (NPC) – sterility check

3.1. Prepare a dilution series from a sample of the extraction medium (e.g., NaCl plus Tween™ 20), without seeds.

3.2. Plate each dilution on the two semi-selective media, spread over the surface and incubate plates with the sample (as in Section 1.4).

4. Examination of the plates

4.1. Examine the NPC and PC plates. There should be no growth on the NPC plates. For the PC plates, the number of bacteria on the dilution plates should be consistent with the dilution, i.e., it should decrease approximately ten-fold with each dilution.

4.2. Examine the sample plates for the presence of typical Xcc/Xcr colonies by comparing them with the PC plates. Dilution plates, prepared from the PC isolates or reference material, should give single colonies with typical morphology. The numbers of colonies on dilution plates prepared from the PC isolates or reference material should be similar on both media.

4.3. After 3–4 days Xcc/Xcr colonies on mFS are small, pale green, mucoid and surrounded by a zone of starch hydrolysis. This zone appears as a halo that may be easier to see with a black background (Figure II.1a). Colonies may show marked variation in size and may be visible on mFS after three days; if not, incubate for an additional day.

4.4. After 3–4 days, Xcc/Xcr colonies on mCS20ABN are pale yellow, mucoid and surrounded by a zone of starch hydrolysis (Figure II.1b). Colonies may show marked variation in size. Depending on the number of colonies present, it may be easier to evaluate plates after three days, before coalescence of starch hydrolysis zones that can make identification of suspect colonies more difficult.

4.5. Incubation of the plates at 4 °C for several hours before recording may result in sharper zones of starch hydrolysis with some starch sources.

4.6. Verify that the plates are readable, according to the Best Practices for Dilution Plating Assays in Seed Health Tests, and record the presence of suspect colonies.

Note: Test results are only valid when all included controls presented in Table II.2 give the expected results.

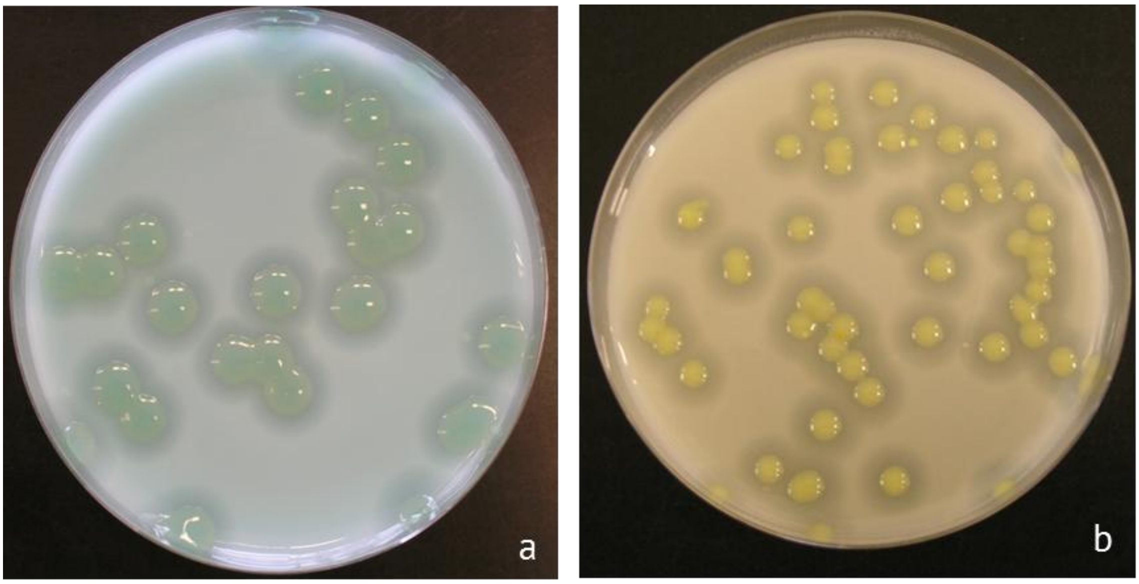

Figure II.1. Plates of mFS (a) and mCS20ABN (b) after five days of incubation at 28 °C showing typical colonies of Xcc surrounded by zones of starch hydrolysis.

5. Confirmation identification of suspect colonies

5.1. Subculture suspect colonies to sectored plates of YDC. To avoid the potential for cross-contamination of isolates, use a new sectored plate for each subsample. The precise numbers of colonies subcultured will depend on the number and variability of suspect colonies on the plate: if present, at least six colonies should be subcultured per subsample.

5.2. Subculture the PC isolate to a sectored plate for comparison.

5.3. Incubate sectored plates for 24–48 hours at 28–30 °C.

5.4. Compare the appearance of growth with the PC. On YDC, Xcc/Xcr colonies are pale yellow and mucoid/fluidal (Figure II.2). The PC isolate(s) or reference material should give colonies with typical morphology on YDC.

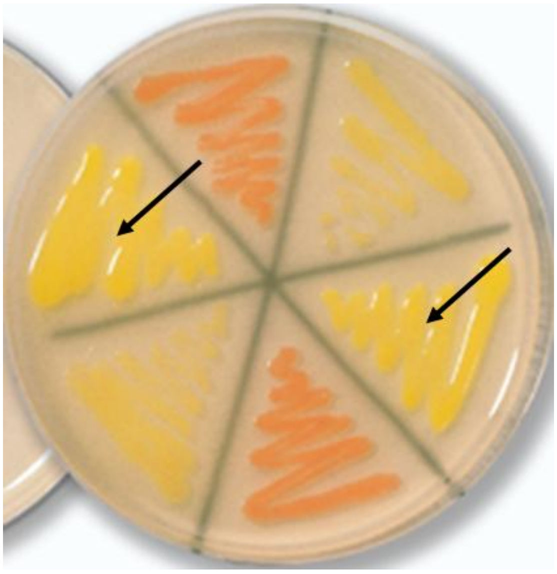

Figure II.2. Typical yellow mucoid growth of isolates of Xcc on a sectored plate of YDC after three days at 28 °C. Suspect cultures are indicated by arrows.

5.5. The identity of the isolates can be confirmed by the qPCR. The pathogenicity of the isolates should be confirmed using known susceptible *Brassica* seedlings by pathogenicity assay.

Note: As non-pathogenic isolates may also be present in seed lots, it is essential to subculture at least the minimum number of suspect colonies specified (six per subsample), and to test the pathogenicity of all *Xanthomonas*-like subcultured isolates by a pathogenicity or qPCR assay.

5.6. Record results for each colony subcultured.

III. SUSPECT COLONY IDENTIFICATION BY qPCR (OPTIONAL STEP)

Materials

- Controls (Table III.1)
- qPCR mix, primers (Table I.1 and III.2) and equipment
- NaCl (0.15 M) (Table II.1)
- NaOH (0.5 M) (Table III.3)
- Tris-HCl (1 M), pH 8.0
- Centrifuge
- Optical Density (OD) meter

Table III.1. Types of controls used.

Control type	Description
Positive process control (PPC)	Freshly prepared suspension of Xcc *and*
	Freshly prepared suspension of Xcr
Negative process control (NPC)	Freshly prepared suspension of non-target colony
Internal amplification control (IAC)	Universal bacterial primers (Wu *et al.*, 2008)
Non template control (NTC)	Nucleic acid-free water

Table III.2. Primer-sequences and references.

Name	Sequence (5’– 3’)	Source
Wu-F	CAA CGC GAA GAA CCT TAC C	Wu *et al.*, 2008
Wu-R	ACG TCA TCC CCA CCT TCC´	
Wu-Pr1	TEXAS RED – ACG ACA ACC ATG CAC CAC CTG – BHQ2	
Wu-Pr2	TEXAS RED – ACG ACA GCC ATG CAG CAC CT – BHQ2	

Table III.3. NaOH 0.5 M.

Compound	Amount/L
NaOH	20 g

1. DNA isolation

The template DNA from single colonies for qPCR can be obtained by several means. The method presented here was used for the validation of this protocol.

1.1. Make a slightly turbid cell suspension (OD_{600} ~ 0.05) in 1.0 mL of sterile NaCl from the suspected cultures on YDC medium and the positive controls (Table III.1). In addition, a non-suspect isolate should be used as a negative process control (NPC).

1.2. Centrifuge bacterial suspensions at 8,000 rpm for 5 min at room temperature. Discard the supernatant and re-suspend the pellet with 500 µL of 0.5 M NaOH.

1.3. Incubate for 10 min at 100 °C.

1.4. Suspensions can be stored at −20 °C until qPCR.

2. qPCR

2.1. Use the Xcc specific primer pairs and probes from Köhl *et al*. (2011) and from Berg *et al*. (2006) (Table I.1).

2.2. Use the universal primers and probes from Wu *et al*. (2008) (Table III.2) to validate the PCR reactions.

2.3. Prepare the reaction mixture (Table III.4 and III.5 for duplex reactions).

2.4. Perform the PCR reactions in a real-time PCR instrument according to the prescribed PCR conditions (Table III.6).

Notes: If different PCR mixtures and amplification programs are used, it is necessary to verify their performance. Validation studies showed that PCR results were more dependent on laboratory conditions than on the PCR protocol itself when different PCR mixes, and amplification products were used in laboratories.

Make sure that the template DNA is at room temperature when added to the mix to prevent temperature linked chemical reactions prior to PCR.

Table III.4. PCR mix qPCR Berg/Wu duplex.

Component	Per reaction (in µL)	Final concentration
DLH153 (10 µM)	1.25	0.5 µM
DLH154 (10 µM)	1.25	0.5 µM
P7 (10 µM, FAM)	0.50	0.2 µM
Wu-F (10 µM)	0.50	0.2 µM
Wu-R (10 µM)	0.50	0.2 µM
Wu-Pr1 (TexRed, 10 µM)	0.50	0.2 µM
Wu-Pr2 (TexRed, 10 µM)	0.50	0.2 µM
PerfeCTa Multiplex qPCR ToughMix (5×)	5.00	1×
PCR grade water	10.00	
Template DNA	5.00	
Total	**25.00**	

Table III.5. PCR mix qPCR Köhl/Wu duplex.

Component	Per reaction (in µL)	Final concentration
XCC-F (10 µM)	0.5	0.2 µM
XCC-R (10 µM)	0.5	0.2 µM
XCC-Pr (10 µM, Vic)	0.5	0.2 µM
Wu-F (10 µM)	0.5	0.2 µM
Wu-R (10 µM)	0.5	0.2 µM
Wu-Pr1 (TexRed, 10 µM)	0.5	0.2 µM
Wu-Pr2 (TexRed, 10 µM)	0.5	0.2 µM
PerfeCTa Multiplex qPCR ToughMix (5×)	5.0	1×
PCR grade water	11.5	
Template DNA	5.0	
Total	**25.0**	

Table III.6. PCR conditions qPCR.

Step	Temperature	Duration
hold	95 °C	10 min
40 cycles	95 °C	15 sec
	60 °C	30 sec

2.5. Cut-off values must be established by each laboratory for their positive and internal amplification controls prior to the assay being used on routine samples. For recommendations on setting cut-off values, see Real-time PCR, an 'indirect' test used for pre-screening in seed health methods. The Cq values of positive controls should consistently be lower than 30. The cut-off Cq value of the internal amplification control (IAC) should be below 35, and the expected range is to be determined by the user based on experimental data.

Note: In the case of universal bacterial primers, positive reactions may occur in non-template controls (NTC) due to the presence of residual DNA in Taq enzyme reagents. The IAC Cq values from reactions on suspect isolates should be at least 3.3 Cq value lower than the IAC Cq values from the NTC reactions.

5. Interpretation and decisions

For interpretation and decision making, the results from both primer sets need to be considered, as described in Table III.7. Test results are only valid when all included controls presented in Table III.1 give the expected results.

Table III.7. Interpretation and decision table for the qPCR.

Köhl qPCR	Berg qPCR	qPCR Result	Follow-up
Positive	Positive	Target DNA for Xcc detected	Pathogenicity test for confirmation
Negative	Positive	Target DNA for Xcr detected	Pathogenicity test for confirmation
Positive	Negative	Inconclusive	Pathogenicity test for confirmation
Negative	Negative	No target DNA for Xcc/Xcr detected	Negative, no follow up

IV. CONFIRMATION BY PATHOGENICITY ASSAY

Materials

- Brassica seedlings: susceptible to all races of the pathogen (e.g., *B. oleracea* 'Wirosa')
- Sterile cocktail sticks (toothpicks) or insect pins
- Controls (Table IV.1)

Table IV.1. Types of controls used.

Control type	Description
Positive process control (PPC)	A known strain of Xcc *and*
	A known strain of Xcr
Negative control (NC)	Sterile cocktail stick or insect pin

1. Pathogenicity assay

1.1. Grow seedlings of a *Brassica* cultivar known to be susceptible to all races of Xcc (e.g., cabbage 'Wirosa', see Vicente *et al.,* 2001) in small pots or modules until at least 3–4 true leaf stage.

1.2. Scrape a small amount of bacterial growth directly from a 24–48 hours YDC culture (e.g., sectored plate) with a sterile cocktail stick or insect pin.

1.3. Inoculate six of the major veins at a point near the leaf edges on the two youngest leaves by stabbing with the cocktail stick or insect pin.

1.4. The number of plants which should be inoculated will depend on the variability of the cultivar and experience of the operator, but one to three plants per isolate should usually be sufficient. It is better to inoculate more isolates with one plant per isolate than fewer isolates with three plants per isolate.

1.5. Inoculate with the positive control isolate and stab with a sterile cocktail stick or insect pin as a negative control. Positive control isolates as well as a negative control should be included in every pathogenicity test (Table IV.1).

1.6. Grow on plants at 20–25 °C.

1.7. Examine plants for the appearance of typical progressive V-shaped, yellow/necrotic lesions with blackened veins caused by the vascular pathogen Xcc after 10–14 days (Figure IV.1a). Symptoms may be visible earlier depending on temperature and the aggressiveness of the isolate. Compare against positive controls.

Note: Test results are only valid when all included controls give the expected result. The positive control isolate should give typical symptoms in the pathogenicity test. It is important to discriminate between the progressive lesions caused by Xcc and the limited dark necrotic lesions at the inoculation site caused by leaf spot Xcr (See Figure IV.1b, Kamoun *et al.,* 1992; Alvarez *et al.,* 1994; Tamura *et al.,* 1994; Vicente *et al.,* 2001; Roberts *et al.,* 2004).

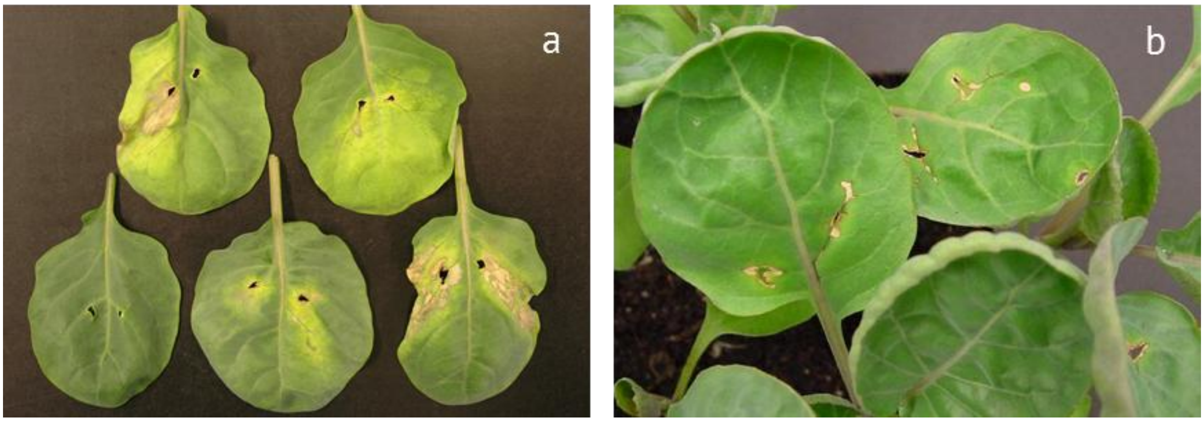

Figure IV.1. Cabbage leaves 7 days post-inoculation with Xcc (a) and Xcr (b). a. The lower left leaf in picture (a) was used as a negative control, typical symptoms of Xcc are black veins, wilting and chlorosis and typical symptoms of Xcr are circular dark spots that become lighter and are occasionally surrounded by a chlorotic halo.

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Annex B. Analytical sensitivity dilution plating results of the Xcc and Xcr dilution series on mCS20ABN medium.

Rep: Repetition, SD: Standard deviation, TNTC: Too numerous to count (>300 colonies).

Target	Series	Rep	Number of CFU				
			Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5
Xcc	**V1**	**1**	TNTC	187	62	10	6
		2	TNTC	203	48	11	4
		3	TNTC	189	46	15	5
	V2	**1**	TNTC	235	56	16	7
		2	TNTC	180	50	16	1
		3	TNTC	238	54	9	4
	V3	**1**	TNTC	184	48	4	1
		2	TNTC	170	52	9	3
		3	TNTC	200	50	9	2
	Average (SD)		TNTC	198.4 (±22.4)	51.8 (±4.7)	11.0 (±3.8)	3.7 (±2.0)
Xcr	**V1**	**1**	TNTC	217	49	15	3
		2	TNTC	232	53	15	4
		3	TNTC	229	58	14	5
	V2	**1**	TNTC	TNTC	80	15	2
		2	TNTC	TNTC	79	14	5
		3	TNTC	TNTC	69	19	2
	V3	**1**	TNTC	TNTC	76	22	2
		2	TNTC	TNTC	86	21	2
		3	TNTC	TNTC	91	12	5
	Average (SD)		TNTC	TNTC	71.2 (±14.0)	16.3 (±3.3)	3.3 (±1.3)

Annex C. Results of CFU of the Xcc and Xcr dilution series spiked into seed extract and NaCl+T after dilution plating on mCS20ABN medium.

Rep: Repetition, SD: Standard deviation.

Back-ground	Spike	Series	Rep	Number of CFU				
				Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5
Lot A	**Xcc**	**V1**	1	158	38	10	1	0
			2	130	38	4	1	0
			3	162	32	8	0	0
		V2	1	124	35	6	3	0
			2	133	41	7	2	0
			3	117	29	6	1	0
		V3	1	154	38	11	4	0
			2	144	27	7	1	0
			3	135	34	2	0	0
		Average (SD)		139.7 (±14.8)	34.7 (±4.4)	6.8 (±2.6)	1.4 (±1.3)	0.0 (±0.0)
	Xcr	**V1**	1	153	36	8	0	1
			2	162	33	11	0	0
			3	174	40	5	0	0
		V2	1	139	47	4	2	1
			2	150	41	9	2	0
			3	181	46	4	3	1
		V3	1	168	42	7	1	0
			2	186	36	7	2	1
			3	173	26	10	3	0
		Average (SD)		165.1 (±14.5)	38.6 (±6.2)	7.2(±2.4)	1.4 (±1.2)	0.4 (±0.5)
	NaCl	**V1**	1	0				
			2	0				
			3	0				
		V2	1	0				
			2	0				
			3	0				
		V3	1	0				
			2	0				
			3	0				
		Average (SD)		0.0 (±0.0)				
NaCl+T	**Xcc**	**V1**	1	146	19	11	1	0
			2	148	26	9	0	0
			3	144	26	7	2	0
		V2	1	114	24	4	1	0
			2	102	19	5	0	0
			3	132	34	2	2	1
		V3	1	93	28	11	0	0
			2	101	27	6	0	0
			3	142	35	5	1	0
		Average (SD)		124.7 (±20.7)	26.4 (±4.9)	6.7 (±2.7)	0.8 (±0.8)	0.1 (±0.3)
	Xcr	**V1**	1	136	28	5	1	0
			2	136	29	5	1	1

Back-ground	Spike	Series	Rep	Number of CFU				
				Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5
			3	138	25	11	1	1
		V2	1	154	35	3	3	0
			2	167	43	12	2	0
			3	173	31	6	1	0
		V3	1	126	26	4	1	0
			2	159	48	6	2	0
			3	161	38	11	1	1
		Average (SD)		150.0 (±15.5)	33.7 (±7.7)	7.0 (±3.3)	1.4 (±0.7)	0.3 (±0.5)
	NaCl	**1**	1	0				
			2	0				
			3	0				
		2	1	0				
			2	0				
			3	0				
		3	1	0				
			2	0				
			3	0				
		Average (SD)		0.0 (±0.0)				

Annex D. Results of CFU of the Xcc and Xcr dilution series spiked into seed extract and NaCl+T after dilution plating on mFS medium.

Rep: Repetition, SD: Standard deviation.

Back-ground	Spike	Series	Rep	Number of CFU				
				Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5
Lot A	**Xcc**	**V1**	1	154	29	10	0	0
			2	154	42	8	0	1
			3	195	66	9	1	0
		V2	1	109	32	9	1	1
			2	132	36	5	1	0
			3	107	51	3	1	0
		V3	1	103	20	5	1	0
			2	119	20	7	1	0
			3	117	21	9	4	1
		Average (SD)		132.2 (±28.5)	35.2 (±14.7)	7.2 (±2.2)	1.1 (±1.1)	0.3 (±0.5)
	Xcr	**V1**	1	130	38	9	2	0
			2	132	31	2	0	0
			3	116	34	5	1	0
		V2	1	153	37	18	2	0
			2	152	42	9	5	0
			3	169	40	11	3	1
		V3	1	152	29	6	3	1
			2	156	42	10	2	0
			3	107	36	3	1	0
		Average (SD)		140.8 (±19.4)	36.6 (±4.3)	8.1 (±4.6)	2.1 (±1.4)	0.2 (±0.4)
	NaCl	**V1**	1	0				
			2	0				
			3	0				
		V2	1	0				
			2	0				
			3	0				
		V3	1	0				
			2	0				
			3	0				
		Average (SD)		0.0 (±0.0)				
NaCl+T	**Xcc**	**V1**	1	144	20	7	1	0
			2	190	26	13	3	0
			3	158	28	13	1	0
		V2	1	108	31	8	2	0
			2	158	20	3	1	0
			3	130	26	10	3	0
		V3	1	102	20	9	0	0
			2	98	31	8	0	0
			3	100	31	5	0	0

Back-ground	Spike	Series	Rep	Number of CFU				
				Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5
		Average (SD)		132.0 (±32.3)	25.9 (±4.3)	8.4 (±3.3)	1.2 (±1.2)	0.0 (±0.0)
	Xcr	**V1**	1	102	37	4	2	1
			2	113	30	8	0	0
			3	117	37	7	2	0
		V2	1	138	33	9	0	0
			2	134	34	10	1	0
			3	148	36	7	0	0
		V3	1	149	32	9	3	1
			2	146	32	7	1	1
			3	129	34	14	0	0
		Average (SD)		130.7 (±13.0)	33.9 (±2.1)	8.3 (±2.2)	1.0 (±1.1)	0.3 (±0.4)
	NaCl	**1**	1	0				
			2	0				
			3	0				
		2	1	0				
			2	0				
			3	0				
		3	1	0				
			2	0				
			3	0				
		Average (SD)		0.0 (±0.0)				

Annex E. Results of CFU of dilution 3 (1:125) for Xcc to evaluate the selectivity.

SD: Standard deviation.

Lot	Spike	Number CFU – CS20ABN		Number CFU – mFS	
		NaCl	Dilution 3	NaCl	Dilution 3
NaCl+T	1	0	3	0	11
	2	0	9	0	4
	3	0	4	0	6
	Average (SD)	0.0 (±0.0)	5.3 (±3.2)	0.0 (±0.0)	7.0 (±3.6)
A	1	0	5	0	5
	2	0	1	0	2
	3	0	3	0	7
	Average (SD)	0.0 (±0.0)	3.0 (±2.0)	0.0 (±0.0)	4.7 (±2.5)
B	1	0	8	0	5
	2	0	11	0	9
	3	0	10	0	5
	Average (SD)	0.0 (±0.0)	9.7 (±1.5)	0.0 (±0.0)	6.3 (±2.3)
C	1	0	12	0	2
	2	0	8	0	9
	3	0	7	0	7
	Average (SD)	0.0 (±0.0)	9.0 (±2.6)	0.0 (±0.0)	6.0 (±3.6)
D	1	0	4	0	3
	2	0	6	0	8
	3	0	6	0	9
	Average (SD)	0.0 (±0.0)	5.3 (±1.2)	0.0 (±0.0)	6.7 (±3.2)
E	1	0	3	0	1
	2	0	5	0	2
	3	0	4	0	1
	Average (SD)	0.0 (±0.0)	4.0 (±1.0)	0.0 (±0.0)	1.3 (±0.6)

Annex F. Results of CFU of the dilution 4 (1:625) for Xcr to evaluate the selectivity.

SD: Standard deviation.

Lot	Spike	Number CFU – mCS20ABN		Number CFU – mFS	
		NaCl	Dilution 4	NaCl	Dilution 4
NaCl-T	**1**	0	1	0	7
	2	0	10	0	8
	3	0	4	0	8
	Average (SD)	0.0 (±0.0)	5.0 (±4.6)	0.0 (±0.0)	7.7 (±0.6)
A	**1**	0	5	0	7
	2	0	5	0	6
	3	0	6	0	7
	Average (SD)	0.0 (±0.0)	5.3 (±0.6)	0.0 (±0.0)	6.7 (±0.6)
B	**1**	0	6	0	13
	2	0	9	0	11
	3	0	8	0	6
	Average (SD)	0.0 (±0.0)	7.7 (±1.5)	0.0 (±0.0)	10.0 (±3.6)
C	**1**	0	7	0	3
	2	0	7	0	7
	3	0	5	0	5
	Average (SD)	0.0 (±0.0)	6.3 (±1.2)	0.0 (±0.0)	5.0 (±2.0)
D	**1**	0	4	0	6
	2	0	5	0	12
	3	0	8	0	6
	Average (SD)	0.0 (±0.0)	5.7 (±2.1)	0.0 (±0.0)	8.0 (±3.5)
E	**1**	0	5	0	6
	2	0	5	0	8
	3	0	8	0	7
	Average (SD)	0.0 (±0.0)	6.0 (±1.7)	0.0 (±0.0)	7.0 (±1.0)

Annex G. SE-qPCR control results for validating the selectivity.

No amp: No amplification, N/A: Not applicable, SD: Standard deviation.

Control	Repetition	Kohl	Berg	Acat
PAC Acat	1	No amp.	No amp.	28.40
	2	No amp.	No amp.	28.19
	3	No amp.	No amp.	28.61
	Average (SD)	N/A	N/A	28.40 (±0.17)
PAC Xcc[a]	1	28.89	No amp.	No amp.
	2	28.21	No amp.	No amp.
	3	28.58	No amp.	No amp.
	Average (SD)	28.56 (±0.28)	N/A	N/A
PAC Xcr	1	No amp.	26.44	No amp.
	2	No amp.	26.76	No amp.
	3	No amp.	26.51	No amp.
	Average (SD)	N/A	26.57 (±0.14)	N/A
NTC	1	No amp.	No amp.	No amp.
	2	No amp.	No amp.	No amp.
	3	No amp.	No amp.	No amp.
	Average (SD)	N/A	N/A	N/A

[a] A synthetic PAC Xcc was used, based on the Kohl fragment only.

Annex H. SE-qPCR results for NaCl for validating the selectivity.

No amp: No amplification, Red cell: Positive signal (Cq<35), SD: Standard deviation.

Lot	Repetition	NaCl		
		Kohl	Berg	Acat
NaCl+T	**1**	No amp.	No amp.	28.49
	2	No amp.	No amp.	28.33
	3	No amp.	No amp.	28.27
	4	No amp.	No amp.	27.62
	5	No amp.	No amp.	27.16
	6	No amp.	No amp.	27.69
Lot A	**1**	No amp.	34.18	27.58
	2	No amp.	No amp.	27.38
	3	36.25	No amp.	27.75
	4	No amp.	No amp.	26.71
	5	No amp.	No amp.	27.15
	6	No amp.	33.01	27.04
Lot B	**1**	36.09	No amp.	26.93
	2	36.75	No amp.	26.90
	3	36.82	31.22	26.92
	4	No amp.	No amp.	27.49
	5	No amp.	33.18	27.45
	6	33.45	No amp.	27.46
Lot C	**1**	No amp.	No amp.	27.31
	2	No amp.	No amp.	27.21
	3	No amp.	No amp.	27.27
	4	No amp.	No amp.	26.94
	5	No amp.	No amp.	26.99
	6	36.24	No amp.	27.13
Lot D	**1**	No amp.	No amp.	27.37
	2	36.12	No amp.	27.30
	3	No amp.	No amp.	27.38
	4	No amp.	No amp.	26.23
	5	No amp.	No amp.	26.06
	6	No amp.	No amp.	26.21
Lot E	**1**	34.08	30.82	26.92
	2	33.84	30.61	27.04
	3	34.53	31.24	26.82
	4	32.71	29.50	26.32
	5	32.21	30.55	26.73
	6	32.92	30.14	26.57
			Average (SD)	27.17 (±0.55)

Annex I. SE-qPCR results for Xcc dilution 3 (1:125) and Xcr dilution 4 (1:625) for validating the selectivity.

No amp: No amplification, N/A: Not applicable, Red cell: Positive signal (Cq<35), SD: Standard deviation.

Lot	Repetition	Xcc			Xcr		
		Kohl	Berg	Acat	Kohl	Berg	Acat
NaCl+T	1	34.31	31.34	28.18	No amp.	No amp.	28.54
	2	36.73	31.90	28.14	No amp.	No amp.	28.58
	3	34.92	31.70	28.33	No amp.	No amp.	28.59
	Average (SD)	35.32 (±1.26)	31.65 (±0.28)	28.22 (±0.10)	N/A	N/A	28.57 (±0.03)
Lot A	1	33.38	32.31	27.56	No amp.	30.67	27.90
	2	33.82	31.84	27.61	No amp.	30.15	27.77
	3	33.58	29.92	27.67	No amp.	31.66	27.71
	Average (SD)	33.59 (±0.22)	31.36 (±1.27)	27.61(±0.06)	N/A	30.83 (±0.77)	27.79 (±0.10)
Lot B	1	32.84	30.98	27.07	No amp.	31.28	27.27
	2	32.91	30.14	26.91	No amp.	31.54	27.08
	3	33.31	30.74	26.63	No amp.	30.66	27.15
	Average (SD)	33.02 (±0.25)	30.62 (±0.43)	26.87 (±0.22)	N/A	31.16 (±0.45)	27.17 (±0.10)
Lot C	1	32.86	30.12	27.06	No amp.	29.76	26.00
	2	32.34	30.82	27.18	No amp.	30.47	26.39
	3	32.19	30.02	26.97	No amp.	30.64	26.82
	Average (SD)	32.46 (±0.35)	30.32 (±0.44)	27.07 (±0.11)	N/A	30.29 (±0.47)	26.40 (±0.41)
Lot D	1	32.86	29.87	27.09	No amp.	30.54	25.80
	2	33.58	30.51	27.39	No amp.	No amp.	25.96
	3	31.78	29.85	27.17	No amp.	No amp.	26.15
	Average (SD)	32.74 (±0.91)	30.08 (±0.38)	27.22 (±0.16)	N/A	30.54	25.97 (±0.18)
Lot E	1	33.46	29.53	27.02	No amp.	29.11	25.91
	2	31.89	29.32	27.40	No amp.	29.31	26.14
	3	32.06	29.69	27.36	34.20	29.06	26.48
	Average (SD)	32.47 (±0.86)	29.51 (±0.19)	27.26 (±0.21)	34.20	29.16 (±0.13)	26.18 (±0.29)
			Average (SD)	27.37 (±0.46)		**Average (SD)**	27.01 (±0.95)