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Detection of *Xanthomonas hortorum* pv. *carotae* in carrot seed using a TaqMan PCR for identification of the suspect bacterial colonies

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ISHI-Veg International Technical Group RBLV – J. Oosterhof (Rijk Zwaan, Netherlands)

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1. INTRODUCTION

Xanthomonas hortorum pv. *carotae* (Xhc) is the causal agent of bacterial blight of carrots (Daucus carota subsp. sativus). It is a common problem wherever carrots are grown, and it can reduce carrot yields and seed quality. Xhc is commonly seed borne and infected seeds are an important inoculum source for the development of bacterial blight in the field (Umesh et al., 1998).

The relationship between the level of carrot seed infected with Xhc and the incidence and severity of carrot bacterial blight was determined in field plots in Davis, California (Umesh et al., 1998). For the environmental conditions that occurred in the area during the study (low rainfall and relative humidity) the threshold of infection for the establishment of Xhc populations on leaves and for the development of carrot bacterial blight was 104 - 105 cfu/gram of seed.

Detection in seed

The most commonly used method in seed health testing laboratories for the detection of *X. hortorum* pv. *carotae* is a seed wash dilution-plating assay. It involves washing seeds in buffer and plating serial dilutions of the extract on a semi-selective medium. For routine testing of carrot seed a combination of two semi-selective media, MKM/MD5A or MKM/mTBM is recommended. It is followed by a conventional PCR assay for confirmation based on work by Asma et al. (2002) showing a PCR assay for confirmation is reliable and quick when compared to a pathogenicity assay.

The detection method for *X. hortorum* pv. *carotae* in carrot seed, a seed wash dilution-plating assay followed by a conventional PCR assay for confirmation validated by ISHI-Veg in 2003 (Asma, 2005). It was approved as an ISTA Rule 7-020 in 2006 (ISTA, 2018) (see https://www.seedtest.org/upload/cms/user/ISTARules2018SHmethod7-020_updated20171109.pdf).

As TaqMan assays are run in a closed system they reduce the risk of cross contamination that conventional gel electrophoresis based PCRs are prone to, and provide a quantitative measurement that increases the speed of obtaining test results. Therefore, the primer sets in a real-time PCR assay (q2) for detecting *X. hortorum* pv. *carotae* published by Temple et al. (2013) and a second TaqMan assay (MVSXhc3) developed by Barnhoorn (2014) were validated by ISHI-Veg for use as an assay for confirming suspect colonies. The gram strain probe-specific based Real-Time PCR developed by Wu et al. (2008) (the 'Wu' primers) was chosen as an internal amplification control (IAC) and the three TaqMan assays were developed as a triplex.

2. OBJECTIVES

The aim of this comparative test was to validate the ability of the triplex (q2, MVSXhc3 and Wu) TaqMan PCR assay to confirm suspect Xhc colonies and replace the conventional PCR in the current ISTA Rule 7-020. Validation was done in accordance to the performance characteristics defined by ISHI-Veg (see Appendix 6.1).

3. METHOD VALIDATION

3.1 GENERAL

Eleven laboratories, representing 5 countries, participated in the comparative test (CT) for determining different performance characteristics and demonstrating the method was fit for purpose.

To determine the stability of the DNA during the period of time when the study was ongoing, Lab 10 performed the assay at 2 different time points, the first at the beginning of the CT on 1 Nov 2018 (coded as Lab 10A) and second at the end of the CT on 14 Jan 2019 (Lab 10B). The gel-based conventional PCR was run only by Lab 10 (Lab 10C). One laboratory performed the assay 2 times using different PCR mixes (Lab 2A and 2B).

The protocol was followed as closely as possible; variants of the reference protocol (in the Fluorochrome or the PCR mix or PCR program used) were properly recorded (see Appendix 6.2). The raw data obtained by the participating labs are presented in Appendix 6.3.

A pre-comparative test was organized to give participating labs an opportunity to gain experience with the newly developed confirmation assay. Ten laboratories from 5 different countries participated in the pre-comparative test. They were provided with DNA from 3 Xhc isolates and 2 non-target Xhc look-alikes from carrot seeds. All 10 labs successfully detected the 3 positives and 2 negatives within the expected range of Quantification cycle (Cq) values. qPCR parameters such as type of qPCR mix, fluorochrome and concentration of ingredients, used in some laboratories were different from those specified in the method but they did not impact the expected results emphasising the robustness of the assay (Oosterhof, 2017).

3.2 ANALYTICAL SPECIFICITY

3.2.1 Definition

The ability of an assay to detect the target(s) pathogens (inclusivity) while excluding non-targets (exclusivity)

3.2.2 Aim

All targets when tested should be found positive, and all non-targets negative.

3.2.3 Experimental approach

The targets and non-targets are presented in Table 1. The targets were 15 Xhc isolates from different origins with 8 in duplicate, resulting in 23 samples. The non-targets were 3 *Xanthomonas* spp. from different crops all in duplicate and resulting in 6 samples, and 12 look-alike (LAL) isolates mostly from carrot seed lots with 9 in duplicate resulting in 21 samples.

Table 1. Target and non-target isolates

category	species	sample(s)	RZ code	origin	year	crop
targeted species	Xhc	8, 38	XHC 12C1	Australia	2010	Carrot leaf
	Xhc	7, 37	XHC 18E3	Netherlands	2003	Carrot seed
	Xhc	5, 35	XHC 25G2	USA	2011	Carrot seed
	Xhc	6, 36	XHC 25G5	USA	2011	Carrot seed
	Xhc	18, 48	XHC 31E5	Germany	2011	Carrot leaf
	Xhc	22	XHC 51B2	USA	2011	Carrot seed

category	species	sample(s)	RZ code	origin	year	crop
	Xhc	28	XHC 62C4	Germany	2015	Carrot seed
	Xhc	13, 43	XHC 62E6	USA	2014	Carrot seed
	Xhc	24	XHC 62F2	Germany	2015	Carrot leaf
	Xhc	29	XHC 63H8	Netherlands	2015	Carrot leaf
	Xhc	4, 44	XHC 63H9	Netherlands	2015	Carrot leaf
	Xhc	23	XHC 8A3	Netherlands	2009	Carrot seed
	Xhc	17, 47	XHC 9A5	Netherlands	2004	Carrot seed
	Xhc	30	XHC 9A5*	Netherlands	2004	Carrot seed
	Xhc	21	XHC41F8	Poland	2012	Carrot leaf
***Xanthomonas* spp.**	X.c. raphani	1, 31	Xca 2	unknown	2011	Brassica
	X.c. campestris	2, 32	Xcc 634	unknown	2011	Brassica
	X.c. vitians	3, 33	Xcvit 2007.1	unknown	2007	Lettuce
Look Alike	unknown	4, 34	LAL 1	unknown	unknown	Carrot seed
	unknown	16, 46	LAL 13	unknown	2017	Carrot seed
	unknown	25	LAL 14D8	ISHI CT2003	2003	Carrot seed
	unknown	19, 49	LAL 18	unknown	2017	Carrot seed
	unknown	10, 40	LAL 3	unknown	2017	Carrot seed
	unknown	11, 41	LAL 4	unknown	2017	Carrot seed
	unknown	12, 42	LAL 5	unknown	2017	Carrot seed
	unknown	15, 45	LAL 6	unknown	2017	Carrot seed
	unknown	20, 50	LAL 6D2	Netherlands	2008	Carrot seed
	unknown	26	LAL 8D2	Netherlands	2008	Carrot seed
	unknown	9, 39	LAL 9	unknown	2017	Carrot seed
	unknown	27	LAL 5C4	Chile	2018	Lettuce

3.2.4 Results

As shown in Tables 2 and 3, all labs generated the expected results; no false positives and no false negatives were reported. Also, the results were comparable to those obtained using the traditional gel-based method performed by Lab 10C (see gel pictures in Appendix 6.4).

Two of the 650 samples were considered invalid because no internal amplification control (IAC) product was generated (sample 33 for lab 6 and sample 16 for lab 10B that repeated the assay at the end of the test). In these very few cases (0.3%), the PCR was repeated for a good result. Also, the gel-based PCR generated one invalid result (sample 16 for lab 10C).

Table 2. Results per lab for each targets and non-targets

(non)-target	category	# results per category found by each participant lab													
		gel	Triplex TaqMan												
		10C	1	2A	2B	3	4	5	6	7	8	9	10A	10B	11
Xhc (23 samples)	#pos	23	23	23	23	23	23	23	23	23	23	23	23	23	23
	#invalid	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	#neg	0	0	0	0	0	0	0	0	0	0	0	0	0	0
X. spp (6 samples)	#pos	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	#invalid	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	#neg	6	6	6	6	6	6	6	5	6	6	6	6	6	6
LAL (21 samples)	#pos	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	#invalid	1	0	0	0	0	0	0	0	0	0	0	0	1	0

(non)-target	category	# results per category found by each participant lab												
		gel	Triplex TaqMan											
		10C	1	2A	2B	3	4	5	6	7	8	9	10A	10B	11
	#neg	20	21	21	21	21	21	21	21	21	21	21	21	20	21

Table 3. Number and percentage of positive, invalid and negative results for targets and non-targets

	Number			Percentage		
Species	**positive**	**invalid**	**negative**	**positive**	**invalid**	**negative**
Xhc	299/299	0/299	0/299	100	0	0
X. spp.	0/78	1/78	77/78	0	1.3	98.7
LAL	0/273	1/273	272/273	0	0.4	99.6

In this study on analytical specificity, 2 samples (46 and 3) were tested in duplicate and the duplicate generated a valid, negative result. The assay is able to detect the targeted pathogen Xhc while excluding the non-target LAL/X. spp. organisms.

3.2.5 Conclusion

All targets were found positive, and all non-targets negative. 0.3% was found invalid because no IAC product was generated, but the duplicate test showed valid, negative results. The validation criteria Analytic specificity is within the specified range.

3.3 ANALYTICAL SENSITIVITY

3.3.1 Definition

Smallest amount of the target pathogen that can be detected i.e. the limit of detection (LOD).

In this method the presence of a suspected colony is confirmed using the TaqMan triplex assay. Sensitivity of the assay is, therefore, not relevant as a pure colony is tested and an excess of target DNA is available in the sample. Even in cases the concentration of the DNA is low or inhibition of the PCR reaction occurs the IAC will not react too.

3.3.2 Aim

The Cq of the positive controls should be clearly positive for all three TaqMan assays developed. In other words, Cq values of the positive controls should be < Cq 25 which corresponds to a sensitivity 100 times greater than that of the assay at the threshold Cq of 32. This is based on a Cq difference of 6.7 for 100x dilutions.

3.3.3 Experimental approach

Five labs used their own positive control with the extraction method described in the protocol.

3.3.4 Results

The Cq of the positive controls are presented in Table 4; all are clearly positive for the three TaqMans. The Cq of q2 ranged from 16.13 to 20.94. All positive controls gave a Cq < 25. The ΔCq with the threshold ranged from 7 (32-24.81) to 18 (= 32-14.49) Cq.

Table 4. Cq values of boiled, bacterial extract from own positive control

lab	q2	MVSXhc3	Wu	conclusion
lab 1	19.16	17.26	17.26	Positive
lab 4	16.40	17.20	14.80	Positive
lab 5	20.94	22.73	24.81	Positive
lab 8	16.78	17.54	16.90	Positive
lab 8	16.13	16.32	15.55	Positive
lab 11	18.54	18.68	16.69	Positive
lab 11	16.41	16.51	14.49	Positive

3.3.5 Conclusion

The sensitivity of the assay is found to be Cq 7 to 18 lower than the threshold setting of Cq 32. The aim was to achieve a sensitivity of at least 100 times, i.e. Cq < 25 in order to reduce invalid results. This goal is achieved.

3.4 SELECTIVITY

3.4.1 Definition

The effect of different seed matrices on the ability of the method to detect target pathogen(s)

The matrix will always be the colony and so there are no other matrices to take into consideration.

3.5 REPEATABILITY

3.5.1 Definition

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

3.5.2 Aim

Based on the qualitative data (binary approach), the results should be 100% repeatable.

3.5.3 Experimental approach

8 Xhc isolates, and 12 non-targets (LAL and X. spp.) were sent in duplicate to each of the 11 labs. As labs 2 and 10 performed the assay twice the test was performed 13 times.

Differences in duplicate runs of the Wu primers were determined for the 156 (12 isolates x 13 labs) non-target isolates. Similarly, differences in the Cq values for the q2 and MVSXhc3 primers for 104 Xhc (8 isolates x 13 labs) were calculated. Cq differences lower than Cq 3 i.e. less by a factor of 10 were expected.

3.5.4 Results

All 104 assays run in duplicate gave positive results for Xhc isolates while 154 of the 156 non-target assays were negative for Xhc isolates in the duplicate runs. The results for the 2 non-target samples were invalid as there was no reaction of the internal amplification control in one of the duplicate runs (see Appendix 6.5 for the differences in the duplicate runs).

In Tables 5A and 5B the Cq differences for duplicate runs are divided into three categories, ΔCq < 1; 1 < ΔCq < 3; and ΔCq > 3).

Table 5A. Classification of Δ Cq values for non-target isolates using **t**he Wu assay

Classification by difference in Cq values	Wu	
	# per cat	%
Δ Cq < 1	142	91.0
1 < ΔCq < 3	11	7.1
ΔCq > 3	1	0.6
Duplicate invalid	2	1.3

Table 5B. Classification of Δ Cq values for target isolates using the q2 and MVSXhc3 assays

Classification by Difference in duplicate runs	q2		MVSXhc3	
	# per cat	%	# per cat	%
ΔCq < 1	100	96.0	101	97.2
1 < ΔCq < 3	3	3.0	2	2.0
ΔCq > 3	1	1.0	1	1.0
Duplicate invalid	0	0.0	0	0.0

The variation in the second run of the Wu primers for non-target LAL 13 exceeded Cq3 just once (ΔCq = 3.77) for lab 7. Similarly, for target Xhc (q2 and MVSXhc3 primers) the variation for the duplicate run exceeded Cq3 just once (0.9%). A careful examination of the sample (Xhc9A5, lab 6) showed that variation in the Wu also exceeds Cq3 suggesting less DNA was added to this specific reaction, most probably due to pipetting errors.

3.5.5 Conclusion

The criterion set for repeatability was met: all 104 samples for Xhc isolates were positive in the duplicated runs. In addition the assays were negative for 154 of the 156 non-target samples in duplicate runs, and 2 were invalid because of no reaction of the IAC in one of the two runs. Invalid data were the consequence of too little DNA in the sample, and in practice the test should be repeated.

3.6 REPRODUCIBILITY

3.6.1 Definition

Degree of similarity in results when the method is performed across labs with replicates of the same subsamples.

3.6.2 Aim

Based on the qualitative data (binary approach), the results should be 100% repeatable by the participating labs.

3.6.3 Experimental approach

The comparative test took place at the end of 2018.

3.6.3.1 Materials

15 Xhc isolates and 15 non-target isolates were sent to each of the 11 labs who performed the test 13 times. Some isolates were prepared in duplicate resulting in 23 samples with target Xhc, 6 samples with non-target *Xanthomonas* spp. and 21 samples with non-target LALs. The lookalikes isolated in Xhc seed tests were suspect based on the morphology but negative using the conventional PCR. The bacterial isolates included in this CT originated from different locations around the world and were collected during routine seed health tests of carrot seed lots using ISTA Rule 7-020, or leaf testing.

DNA was extracted from the 50 isolates (23 target Xhc, 6 non-target *Xanthomonas* spp. and 21 LAL) using a Qiagen kit and coded from 1 to 50 randomly. Each participant received one set of these 50 genomic DNA samples. In addition tubes containing genomic DNA of both Xhc and *Xanthomonas campestris campestris* were also provided to each lab to use as a Positive Control (PC) and Negative Control (NC), respectively. A Non Template Control (NTC) was also provided. Each participating lab was also requested to include at least one Xhc positive boiled bacterial extract from their own collection to demonstrate the effectiveness of the triplex TaqMan PCR assay on the range of Xhc isolates. Instructions for receiving and storing samples, and avoiding cross contamination are also provided to each participating lab.

3.6.3.2 Methods

In this comparative test the organizing laboratory performed both the conventional PCR described in ISTA method 7-020, and the triplex TaqMan PCR presented below in Table 6 to 8.

Table 6. TaqMan PCR Primer and probe sequences

Name	Dye	Sequence 5'-3'	Quencher	Reference
MVSXhc3-F		CCAAAGCAGTCGCAAACTTGA		Barnhoorn 2014
MVSXhc3-R		AATTGCGGATTCCCAACAAA		
MVSXhc3-P	Vic*	TGGCCCTAAGCTTCAA	MGB(NFQ)*	
q2-F		GCATGAAGGCAATACAGCG		Temple et al., 2013
q2-R		CGATCCAGCTGATGTTCTCCGAA		
q2-P	FAM*	TCAAGCTCAGACGAAACCGGCGTC	BHQ1*	
Wu-F		CAACGCGAAGAACCTTACC		Wu et al., 2008
Wu-R		ACGTCATCCCCACCTTCC		
Wu-P1	TxRd*	ACGACAACCATGCACCACCTG	BHQ2*	
Wu-P2	TxRd*	ACGACAGCCATGCAGCACCT	BHQ2*	

* If the Fluorochrome or quencher used by participating labs differs from what is indicated in the table, it should be recorded in the results sheet

Table 7. Pipetting scheme q2, MVSXhc3, Wu triplex PCR

Component	Final conc.	1x (volume in µl)
q2-F (20 µM)	400 nM	0.5
q2-R (20 µM)	400 nM	0.5
q2-P (20 µM, FAM*)	200 nM	0.25
MVSXhc3-F (20 µM)	900 nM	1.125
MVSXhc3-R (20 µM)	900 nM	1.125
MVSXhc3-P (20 µM, Vic*)	250 nM	0.3125
Wu-F (20µM)	200 nM	0.25

Wu-R (20µM)	200 nM	0.25
Wu-P1 (20µM,TxRd*)	200 nM	0.25
Wu-P2 (20µM,TxRd*)	200 nM	0.25
ABI Gene Expression Master mix (2x)*	1X	12.5
milliQ		5.6875
DNA		2.0
Total Volume		25.0

* Labs were free to use a master mix or Fluorochrome of their choice but this had to be indicated in the remarks field on the results sheet

Table 8. TaqMan PCR program

Time	Temperature	# cycles
10 min.	95^0C	
15 sec.	95^0C	40x
30 sec.	60^0C	

Note: Labs were free to use the TaqMan program of their choice but this had to be indicated in the remarks field on the results sheet

Other laboratories tested only the newly developed triplex TaqMan PCR assay. The Wu primers and probes (Wu et al., 2008) were included in the triplex assay as an Internal Amplification Control (IAC) to confirm bacterial DNA presence. They have been developed using sequences from the 16S rDNA region that are conserved in almost all bacteria, and therefore, a positive amplification is expected with the Wu assay for every sample tested containing bacterial DNA. Participants were instructed to follow the protocols as closely as possible and the deviations in Fluorochrome, PCR mix or PCR program were expected to be properly recorded in the results.

3.6.3.3 Critical points

- Prevent DNA contamination
- Store extracted DNA at -20°C in case further analysis is required

3.6.3.4 Interpretation of the TaqMan assay (performed by all participating labs)

All raw data including interpretation of the results were recorded on the result form.

All the samples were first scored in relation to the Cq-value determined for the Non-Template Control (NTC). As the Wu assay reacts with residual microbial DNA present in the polymerase in qPCR master mixes, in some cases it may have led to a rather low Cq-value of 28 for the NTC. To determine if a sample was properly prepared, the Cq-value of the Wu assay in Xhc negative samples should have been at least 3.3 Cq values lower than the NTC. In other words, the sample needed to contain almost 10 times more microbial DNA than was present in the NTC.

When the difference in the Cq-value of the NTC and the sample was ⩽ 3.3, a new sample had to be tested and compared with a new NTC.

When the DNA of the target pathogens was high, competition was observed in the pathogen-specific (q2 and MVSXhc3) and the Wu assays. In such cases the interpretation scheme presented in Table 9 was used.

Table 9. TaqMan scoring interpretation scheme

Assay	Result

q2	MVSXhc3	Wu	
Cq ⩽32	Not applicable	Not applicable	Xhc positive
Not applicable	Cq ⩽32	Not applicable	Xhc positive
Cq>32	Cq>32	Cq ⩽32	Xhc negative
Cq>32	Cq>32	Cq>32	invalid

Note: Scores can only be given when the Cq-values for Wu assay in the samples are at least 3.3 Cq values lower than that found in the NTC

3.6.3.5 Interpretation of the conventional gel-based PCR assays (performed by 1 laboratory)

In the current ISTA Rule 7-020 the scoring specifications of the conventional PCR are presented. The fragment sizes of the DNA bands on the agarose gel is described below along with the decision, viz. two bands (specific and universal) = positive identification and one band (universal) = negative identification of Xhc.

3.6.4 Results

All 15 Xhc isolates were found positive and all 15 non-targets were found negative in the 13 tests run.

3.6.5 Conclusion

The reproducibility meets the criteria set viz. the samples with the target were found to be positive and the samples with non-targets were found negative.

3.7 ROBUSTNESS

3.7.1 Definition ISHI-Veg guidelines

Not described in the ISHI-Veg guidelines

3.7.2 Aim

Repeatability of the TaqMan triplex assay should be 100%.

3.7.3. Experimental approach

Labs were free to differ from the prescribed protocol by using their own fluorochromes, master mixes and PCR programs (see Appendix 6.2). A comparison of lab performances gave information on the robustness of the assay. Quantitative data (or continuous data) on the Cq values obtained for the samples with the target were analyzed by pooling the result for each isolate in a boxplot.

3.7.4 Results

Similar to the results obtained for repeatability in section 3.5, all 15 Xhc isolates and 15 non-targets were found positive and negative, respectively in the 13 tests. In Figure 2 two BoxPlots are presented: one for the q2 primer set and the other for MVSXhc3. Each bar shows Cq values for each Xhc isolate obtained by the different labs. The x-axis identifies the code of the Xhc-isolate (e.g. 12C1), and the number of samples tested (13 when tested in 13 labs, or 26 when tested in duplicate in 13 labs) is in brackets.

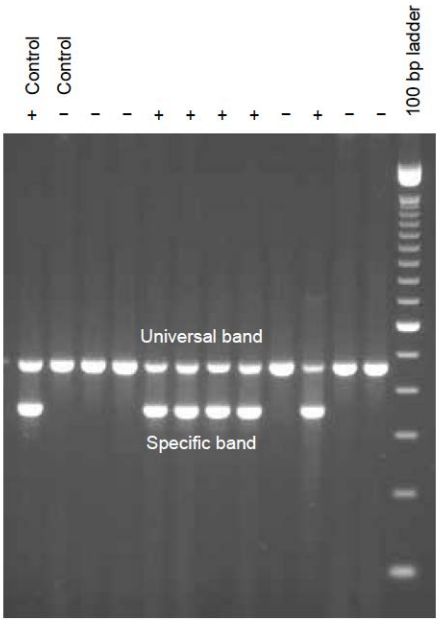

Figure 1. Agarose gel showing *Xanthomonas hortorum* pv. *carotae* specific products of 355 bp and universal bacterial products of 441 bp. Picture copied from ISTA's protocol.

The data largely fits into the bars with the exception of three (red dots) outliers (e.g. isolate 9A5 in sample 17 tested by lab 6). From a close look at these data points, it was clear the Cq of all the three TaqMans in this sample exceeded the threshold (Cq obtained in the TaqMans q2, MVSXhc3, Wu are 29.77, 30.98 and 27.03, respectively) probably due to pipetting error of DNA into the mix).

All labs performed well and no false positives or false negatives were found.

3.7.5 Conclusion

The TaqMan assay was shown to be robust.

4. CONCLUSION

The triplex TaqMan for the confirmation of suspected colonies is fit for purpose. All validation criteria are fulfilled:

- Analytic specificity: All targets were found positive and all non-targets negative. 0.3% was found invalid because no IAC product was generated, but the duplicate showed valid, negative results. The prescribed method showed good performance across the 11 participating labs and no false positives or negatives were found.
- Analytic sensitivity: The aim was to achieve a sensitivity of at least 100 times below the threshold setting, i.e. Cq < 25, in order to reduce invalid results. The sensitivity of the assay is found to be Cq 7 to 18 lower than the threshold setting of Cq 32.
- Selectivity was not relevant as a pure colony was tested. The matrix is always the colony itself and does not change.

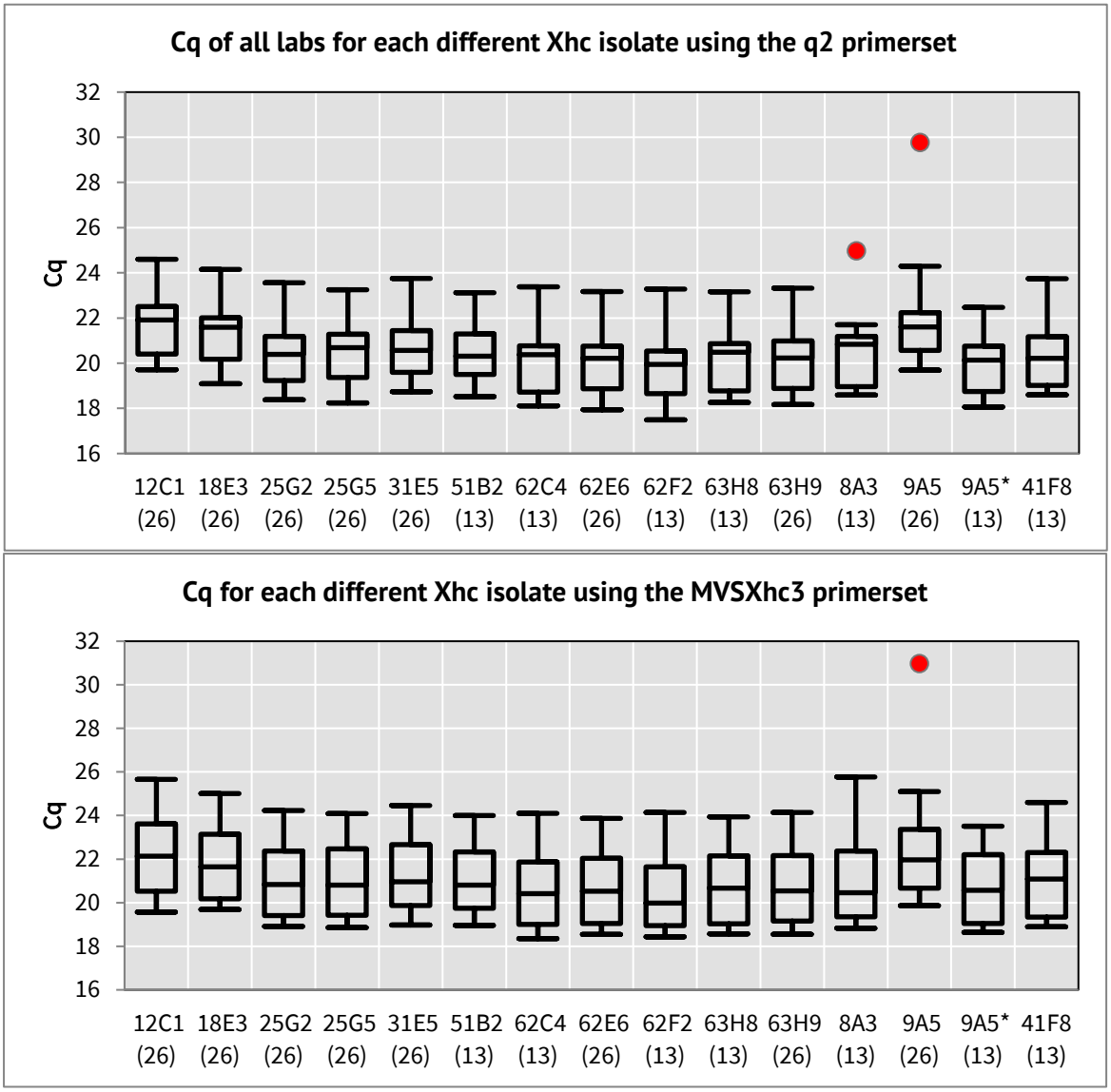

Figure 2. Boxplot of Cq data from all labs for each isolate tested using the q2 and MVSXhc3 primer sets.

- Repeatability: All 104 targets were positive in the duplicate runs, 154 of the 156 non-targets were negative in the runs, and 2 non-targets were invalid in one because of no reaction of the IAC. Invalid data are the consequence of too little DNA in the sample and in practice should be repeated.
- Reproducibility: the criterion was met; in all tests the targets were found positive and all non-targets negative.
- Robustness: Deviations in Fluorochrome, PCR mix or PCR program as recorded in Appendix 6.2 did not have a significant effect on the final results showing the robustness of the assay.

This validation study shows the multiplex TaqMan can be used to generate reliable results and is fit for purpose.

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6. Appendices

Appendix 6.1 ISHI-Veg Method Performance Characteristics

Performance Criteria	Characteristics
Analytical specificity of an assay	The ability of an assay to detect the target(s) pathogens (inclusivity) while excluding non-targets (exclusivity)
Analytical sensitivity	Smallest amount of the target pathogen that can be detected i.e. the limit of detection (LOD)
Selectivity	The effect of different seed matrices on the ability of the method to detect target pathogen(s)
Repeatability	Degree of similarity in results of replicates of the same seed lots when the method is performed with minimal variations in a single lab
Reproducibility	Degree of similarity in results when the method is performed across labs with replicates of the same subsamples
Diagnostic performance	The ability of the method to detect target pathogens in known infected seed samples while excluding non-target organisms in known healthy seed samples
Post-implementation surveillance	After a method has been shown to be fit for purpose evaluating its performance over time to ensure it is performing as intended

Source: ISHI-Veg Guidelines for the Validation of Seed Health Tests, Version 1, May 2018.

Note: Version 2 dated May 2020 that is currently in force doesn't include Post-implementation surveillance.

Appendix 6.2 Materials and methods used by the participating labs (the cells in green refer to the protocol proposed in the test plan)

Lab	Date recd. & test date	Fluorochrome			PCR Mix	PCR program
		MVSXhc3	q2	Wu		
Reference protocol		**VIC**	**FAM**	**Texas Red**	**ABI Gene Expression Master mix**	**10 min. 95^{0}C, 15 sec; 95^{0}C, 30 sec 60^{0}C: 40X**
1	5-Nov-2018 6-Nov-2018	FAM	HEX	Cy5	Roche FastStart Essential DNA Probe Master	Activate: 95^{0}C/10min, Cycling: 95^{0}C/15sec-60^{0}C/30sec 45X
2A	16-Nov-2018 26-Nov-2018	HEX-BHQ2	FAM-BHQ1	Cy5-BHQ2	ABI Gene Expression Master mix	Denature: 95^{0}C/10min PCR: 95^{0}C/15sec-60^{0}C/30sec
2B	16-Nov-2018 27-Nov-2018	HEX-BHQ2	FAM-BHQ1	Cy5-BHQ2	TAKARA Premix Ex Taq (probe qPCR)	Denature: 95^{0}C/30sec, PCR: 95^{0}C/5sec-60^{0}C/30sec
3	21-Nov-2018 27-Nov-2018	FAM-MGBNFQ	Cy5-ZENIABLK	HEX-ZENIABLK	Perfect Multi qPCR ToughMix (cat.89497-280)	10 min. 95^{0}C; 15 sec. 95^{0}C; 30 sec 60^{0}C 40X
4	13-Nov-2018 03-Dec-2018	NED-MGBNFQ	FAM	VIC	Taq PATH qPCR master mix from Life Technology	10 min. 95^{0}C; 15 sec. 95^{0}C; 30 sec 60^{0}C 40X
5	08-Nov-2018 30-Nov-2018	VIC	FAM	Texas Red	PERFECTA Multiplex qPCR Toughmix 5X	10 min. 95^{0}C; 15 sec. 95^{0}C; 1 min 60^{0}C 40X
6	01-Nov-2018 19-Dec-2018	Yakima Yellow	ZEN-Iowa Black FQ	Iowa Black FQ	Quantabio PerfeCTa qPCR ToughMix (5x) no Rox	10 min. 95^{0}C; 15 sec. 95^{0}C; 30 sec 60^{0}C 40X
7	01-Nov-2018 14-Dec-2018	VIC	FAM	Texas Red	TaqMan™ Fast Universal PCR Master Mix (2X), No AmpErase™ UNG (ABI)	10 min. 95^{0}C; 15 sec. 95^{0}C; 30 sec 60^{0}C 40X
8	02-Nov-2018 14-Jan-2018	FAM-MGB	ABY-QSY	HEX-BHQ1	PerfeCTa Multiplex qPCR ToughMix (QuantaBio)	10 min. 95^{0}C; 15 sec. 95^{0}C; 30 sec 60^{0}C 40X
9	30-Nov-2018 08-Jan-2018	VIC	FAM	Texas Red	ABI Gene Expression Master mix	10 min. 95^{0}C; 15 sec. 95^{0}C; 30 sec 60^{0}C 40X
10A	01-Nov-2018 01-Nov-2018	VIC	FAM	Texas Red	ABI Gene Expression Master mix	10 min. 95^{0}C; 15 sec. 95^{0}C; 30 sec 60^{0}C 40X
10B	01-Nov-2018 14-Jan-2018	VIC	FAM	Texas Red	ABI Gene Expression Master mix	10 min. 95^{0}C; 15 sec. 95^{0}C; 30 sec 60^{0}C 40X
11	18-Nov-2018 06-Feb-2018	VIC	FAM	Texas Red	SsoAdvanced Universal Probes Supermix (BIO-RAD)	10 min. 95^{0}C; 15 sec. 95^{0}C; 30 sec 60^{0}C 40X

Appendix 6.3 Results obtained by each participating lab (LAL=look-alike, X.spp=*Xanthomonas* spp. other than Xhc, Xhc=*Xanthomonas hortorum carotae*, PPC= Positive process control, NPC = Negative process control, NTC= Negative template control; PT=position of tube during CT)

Sample code	Isolate code	expected isolate	PT	lab 1				lab 2A				lab 2B			
				q2	MVSXhc3	Wu	Result	q2	MVSXhc3	Wu	Result	q2	MVSXhc3	Wu	Result
PPC		PPC	tube	20.85	19.40	17.75	Positive	20.50	22.84	17.95	Positive	22.50	23.37	19.41	Positive
NPC		NPC	tube	-	-	22.63	Negative	-	-	29.17	Negative	-	-	25.42	Negative
NTC		NTC	tube	-	-	-	OK	-	-	-	OK	-	-	-	OK
1	XCA	X.spp	A1	-	-	16.26	Negative	-	-	22.91	Negative	-	-	19.12	Negative
2	XCC	X.spp	B1	-	-	18.47	Negative	-	-	23.35	Negative	-	-	22.28	Negative
3	XCVIT	X.spp	C1	-	-	18.55	Negative	-	-	23.62	Negative	-	-	22.17	Negative
4	LAL 1	LAL	D1	-	-	17.05	Negative	-	-	21.71	Negative	-	-	21.00	Negative
5	XHC 25G2	Xhc	E1	20.94	19.22	18.34	Positive	20.27	22.52	-	Positive	23.56	24.23	20.86	Positive
6	XHC 25G5	Xhc	F1	21.01	19.27	18.54	Positive	20.61	22.49	-	Positive	23.00	23.74	20.52	Positive
7	XHC 18E3	Xhc	G1	21.69	20.01	19.01	Positive	21.49	23.47	-	Positive	24.15	25.01	21.43	Positive
8	XHC 12C1	Xhc	H1	22.43	20.55	20.16	Positive	21.80	23.86	-	Positive	24.60	25.66	21.80	Positive
9	LAL 9	LAL	A2	-	-	18.75	Negative	-	-	24.10	Negative	-	-	22.42	Negative
10	LAL 3	LAL	B2	-	-	19.63	Negative	-	-	24.32	Negative	-	-	22.11	Negative
11	LAL 4	LAL	C2	-	-	19.13	Negative	-	-	23.79	Negative	-	-	21.32	Negative
12	LAL 5	LAL	D2	-	-	19.64	Negative	-	-	25.53	Negative	-	-	24.22	Negative
13	XHC 62E6	Xhc	E2	20.59	18.97	18.25	Positive	19.92	22.10	-	Positive	23.17	23.87	20.47	Positive
14	XHC 63H9	Xhc	F2	20.54	18.95	18.37	Positive	20.14	22.55	-	Positive	23.27	24.13	20.55	Positive
15	LAL 6	LAL	G2	-	-	18.08	Negative	-	-	22.98	Negative	-	-	22.20	Negative
16	LAL 13	LAL	H2	-	-	18.26	Negative	-	-	23.04	Negative	-	-	21.44	Negative
17	XHC 9A5	Xhc	A3	21.88	20.24	19.71	Positive	21.44	23.42	-	Positive	24.29	25.10	21.73	Positive
18	XHC 31E5	Xhc	B3	20.96	19.29	18.86	Positive	20.58	22.74	-	Positive	23.60	24.46	20.72	Positive
19	LAL 18	LAL	C3	-	-	17.86	Negative	-	-	22.53	Negative	-	-	21.00	Negative
20	LAL 6D2	LAL	D3	-	-	20.13	Negative	-	-	22.67	Negative	-	-	23.67	Negative
21	XHC41F8	Xhc	E3	20.81	19.32	17.69	Positive	20.21	22.33	-	Positive	23.74	24.60	20.27	Positive
22	XHC 51B2	Xhc	F3	20.74	18.96	18.69	Positive	20.31	22.54	-	Positive	23.12	24.00	20.58	Positive

Sample code	Isolate code	expected isolate	PT	lab 1				lab 2A				lab 2B			
				q2	MVSXhc3	Wu	Result	q2	MVSXhc3	Wu	Result	q2	MVSXhc3	Wu	Result
23	XHC 8A3	Xhc	G3	20.83	19.11	18.81	Positive	21.36	23.44	-	Positive	24.98	25.77	21.91	Positive
24	XHC 62F2	Xhc	H3	20.17	18.45	18.07	Positive	19.95	22.14	-	Positive	23.28	24.14	20.12	Positive
25	LAL 14D8	LAL	A4	-	-	17.28	Negative	-	-	22.28	Negative	-	-	18.40	Negative
26	LAL 8D2	LAL	B4	-	-	18.67	Negative	-	-	22.42	Negative	-	-	20.86	Negative
27	LAL XCVIT 5C4	LAL	C4	-	-	16.67	Negative	-	-	21.50	Negative	-	-	18.91	Negative
28	XHC 62C4	Xhc	D4	20.60	19.00	18.25	Positive	20.37	22.35	-	Positive	23.38	24.10	20.68	Positive
29	XHC 63H8	Xhc	E4	20.48	19.01	18.55	Positive	20.55	22.40	-	Positive	23.16	23.94	20.71	Positive
30	XHC 9A5*	Xhc	F4	20.39	18.83	18.51	Positive	20.13	22.38	-	Positive	22.47	23.51	20.05	Positive
31	XCA	X.spp	G4	-	-	15.79	Negative	-	-	21.03	Negative	-	-	18.69	Negative
32	XCC	X.spp	H4	-	-	18.14	Negative	-	-	21.82	Negative	-	-	21.86	Negative
33	XCVIT	X.spp	A5	-	-	18.43	Negative	-	-	23.30	Negative	-	-	21.66	Negative
34	LAL 1	LAL	B5	-	-	17.02	Negative	-	-	22.11	Negative	-	-	20.80	Negative
35	XHC 25G2	Xhc	C5	20.88	19.18	18.45	Positive	20.51	22.48	-	Positive	23.08	23.87	20.64	Positive
36	XHC 25G5	Xhc	D5	20.93	19.27	18.15	Positive	20.75	23.33	-	Positive	23.25	24.09	20.84	Positive
37	XHC 18E3	Xhc	E5	21.82	20.03	19.54	Positive	21.70	23.69	-	Positive	23.88	24.88	21.43	Positive
38	XHC 12C1	Xhc	F5	22.04	20.17	20.09	Positive	22.02	24.07	-	Positive	24.56	25.45	22.01	Positive
39	LAL 9	LAL	G5	-	-	18.91	Negative	-	-	23.68	Negative	-	-	22.20	Negative
40	LAL 3	LAL	H5	-	-	19.29	Negative	-	-	24.20	Negative	-	-	21.51	Negative
41	LAL 4	LAL	A6	-	-	19.29	Negative	-	-	23.94	Negative	-	-	21.90	Negative
42	LAL 5	LAL	B6	-	-	19.70	Negative	-	-	24.54	Negative	-	-	22.86	Negative
43	XHC 62E6	Xhc	C6	20.60	18.97	17.78	Positive	20.50	22.88	-	Positive	22.70	23.64	20.06	Positive
44	XHC 63H9	Xhc	D6	20.72	19.05	18.16	Positive	20.31	22.39	-	Positive	23.32	24.14	20.63	Positive
45	LAL 6	LAL	E6	-	-	18.20	Negative	-	-	22.96	Negative	-	-	22.08	Negative
46	LAL 13	LAL	F6	-	-	18.26	Negative	-	-	22.53	Negative	-	-	21.26	Negative
47	XHC 9A5	Xhc	G6	21.70	19.87	19.71	Positive	21.51	23.69	-	Positive	23.93	24.87	21.42	Positive
48	XHC 31E5	Xhc	H6	20.98	19.09	19.05	Positive	20.54	22.69	-	Positive	23.75	24.45	20.72	Positive
49	LAL 18	LAL	A7	-	-	18.65	Negative	-	-	22.81	Negative	-	-	21.84	Negative

Sample code	Isolate code	expected isolate	PT	lab 1				lab 2A				lab 2B			
				q2	MVSXhc3	Wu	Result	q2	MVSXhc 3	Wu	Result	q2	MVSXhc3	Wu	Result
50	LAL 6D2	LAL	B7	-	-	19.65	Negative	-	-	21.83	Negative	-	-	20.96	Negative

Sample code	Isolate code	expected isolate	PT	lab 3				lab 4				lab 5			
				q2	MVSXhc3	Wu	Result	q2	MVSXhc3	Wu	Result	q2	MVSXhc3	Wu	Result
PPC		PPC	tube	20.75	19.77	18.81	Positive	21.60	22.50	18.60	Positive	19.23	21.24	18.03	Positive
NPC		NPC	tube	-	-	24.47	Negative	-	-	23.90	Negative	-	-	23.79	Negative
NTC		NTC	tube	-	-	36.41	OK	-	-	31.20	OK	-	-	25.13	not OK
1	XCA	X.spp	A1	-	-	18.03	Negative	-	-	17.50	Negative	-	-	16.26	Negative
2	XCC	X.spp	B1	-	-	19.45	Negative	-	-	19.10	Negative	-	-	18.94	Negative
3	XCVIT	X.spp	C1	-	-	20.15	Negative	-	-	19.90	Negative	-	-	19.09	Negative
4	LAL 1	LAL	D1	-	-	18.53	Negative	-	-	18.00	Negative	-	-	17.83	Negative
5	XHC 25G2	Xhc	E1	21.30	20.32	19.93	Positive	21.80	22.50	19.40	Positive	19.36	21.09	19.14	Positive
6	XHC 25G5	Xhc	F1	21.29	20.35	19.94	Positive	22.00	22.60	19.50	Positive	19.51	21.19	19.57	Positive
7	XHC 18E3	Xhc	G1	22.12	21.14	20.76	Positive	22.60	23.20	20.10	Positive	20.32	21.92	20.14	Positive
8	XHC 12C1	Xhc	H1	22.45	21.43	21.21	Positive	23.00	23.70	20.60	Positive	21.55	22.86	21.07	Positive
9	LAL 9	LAL	A2	-	-	19.91	Negative	-	-	19.80	Negative	-	-	19.65	Negative
10	LAL 3	LAL	B2	-	-	20.70	Negative	-	-	20.30	Negative	-	-	20.28	Negative
11	LAL 4	LAL	C2	-	-	20.56	Negative	-	-	20.30	Negative	-	-	20.04	Negative
12	LAL 5	LAL	D2	-	-	21.20	Negative	-	-	20.70	Negative	-	-	20.44	Negative
13	XHC 62E6	Xhc	E2	20.95	20.08	19.88	Positive	21.40	22.10	19.10	Positive	18.74	20.67	18.67	Positive
14	XHC 63H9	Xhc	F2	21.05	20.17	19.91	Positive	21.50	22.30	19.20	Positive	18.77	20.77	18.50	Positive
15	LAL 6	LAL	G2	-	-	19.67	Negative	-	-	19.30	Negative	-	-	19.08	Negative
16	LAL 13	LAL	H2	-	-	19.78	Negative	-	-	19.30	Negative	-	-	19.26	Negative
17	XHC 9A5	Xhc	A3	22.25	21.20	20.97	Positive	22.70	23.30	20.30	Positive	20.52	22.07	20.19	Positive
18	XHC 31E5	Xhc	B3	21.44	20.48	20.32	Positive	21.80	22.60	19.50	Positive	19.51	21.33	19.58	Positive
19	LAL 18	LAL	C3	-	-	19.42	Negative	-	-	18.70	Negative	-	-	18.51	Negative
20	LAL 6D2	LAL	D3	-	-	21.25	Negative	-	-	19.70	Negative	-	-	20.34	Negative

Sample code	Isolate code	expected isolate	PT	lab 3				lab 4				lab 5			
				q2	MVSXhc3	Wu	Result	q2	MVSXhc3	Wu	Result	q2	MVSXhc3	Wu	Result
21	XHC41F8	Xhc	E3	21.01	20.22	18.92	Positive	21.40	22.30	18.40	Positive	19.01	21.08	17.94	Positive
22	XHC 51B2	Xhc	F3	21.29	20.34	19.99	Positive	21.70	22.50	19.30	Positive	19.40	21.20	19.63	Positive
23	XHC 8A3	Xhc	G3	21.19	20.26	19.92	Positive	21.70	22.50	19.40	Positive	18.59	20.46	18.32	Positive
24	XHC 62F2	Xhc	H3	20.54	19.56	19.38	Positive	21.10	21.90	18.80	Positive	18.49	20.24	18.85	Positive
25	LAL 14D8	LAL	A4	-	-	19.08	Negative	-	-	18.00	Negative	-	-	18.60	Negative
26	LAL 8D2	LAL	B4	-	-	19.75	Negative	-	-	19.40	Negative	-	-	19.80	Negative
27	LAL XCVIT 5C4	LAL	C4	-	-	18.24	Negative	-	-	18.20	Negative	-	-	17.68	Negative
28	XHC 62C4	Xhc	D4	20.85	19.93	19.68	Positive	21.40	22.20	19.10	Positive	18.72	20.71	18.78	Positive
29	XHC 63H8	Xhc	E4	20.91	20.02	19.81	Positive	21.30	22.20	19.10	Positive	18.77	20.82	18.62	Positive
30	XHC 9A5*	Xhc	F4	20.54	19.99	19.78	Positive	21.30	22.20	19.10	Positive	18.63	20.68	18.85	Positive
31	XCA	X.spp	G4	-	-	17.68	Negative	-	-	17.20	Negative	-	-	16.25	Negative
32	XCC	X.spp	H4	-	-	19.25	Negative	-	-	19.00	Negative	-	-	18.73	Negative
33	XCVIT	X.spp	A5	-	-	19.87	Negative	-	-	19.60	Negative	-	-	19.15	Negative
34	LAL 1	LAL	B5	-	-	18.33	Negative	-	-	17.80	Negative	-	-	17.29	Negative
35	XHC 25G2	Xhc	C5	21.18	20.29	19.91	Positive	21.60	22.40	19.30	Positive	19.18	21.02	18.76	Positive
36	XHC 25G5	Xhc	D5	21.33	20.41	20.10	Positive	21.80	22.60	19.50	Positive	19.28	21.28	19.37	Positive
37	XHC 18E3	Xhc	E5	22.12	21.15	20.90	Positive	22.50	23.20	20.10	Positive	20.14	21.95	20.09	Positive
38	XHC 12C1	Xhc	F5	22.53	21.57	21.48	Positive	23.30	24.00	21.10	Positive	20.52	22.21	20.76	Positive
39	LAL 9	LAL	G5	-	-	20.20	Negative	-	-	19.80	Negative	-	-	18.90	Negative
40	LAL 3	LAL	H5	-	-	20.76	Negative	-	-	20.40	Negative	-	-	20.19	Negative
41	LAL 4	LAL	A6	-	-	20.60	Negative	-	-	20.10	Negative	-	-	19.98	Negative
42	LAL 5	LAL	B6	-	-	21.05	Negative	-	-	21.40	Negative	-	-	20.62	Negative
43	XHC 62E6	Xhc	C6	20.78	19.94	19.56	Positive	21.60	22.40	19.30	Positive	18.89	20.88	18.65	Positive
44	XHC 63H9	Xhc	D6	20.80	19.98	19.51	Positive	21.40	22.20	19.10	Positive	18.92	20.92	18.70	Positive
45	LAL 6	LAL	E6	-	-	19.60	Negative	-	-	19.10	Negative	-	-	19.25	Negative
46	LAL 13	LAL	F6	-	-	19.52	Negative	-	-	19.30	Negative	-	-	19.24	Negative
47	XHC 9A5	Xhc	G6	22.12	21.14	20.79	Positive	22.80	23.50	20.40	Positive	20.34	21.96	19.55	Positive

Sample code	Isolate code	expected isolate	PT	lab 3				lab 4				lab 5			
				q2	MVSXhc3	Wu	Result	q2	MVSXhc3	Wu	Result	q2	MVSXhc3	Wu	Result
48	XHC 31E5	Xhc	H6	21.39	20.45	20.30	Positive	21.90	22.70	19.70	Positive	19.12	20.82	18.61	Positive
49	LAL 18	LAL	A7	-	-	19.46	Negative	-	-	18.80	Negative	-	-	19.00	Negative
50	LAL 6D2	LAL	B7	-	-	21.25	Negative	-	-	19.90	Negative	-	-	20.85	Negative

Sample code	Isolate code	expected isolate	PT	lab 6				lab 7				lab 8			
				q2	MVSXhc3	Wu	Result	q2	MVSXhc3	Wu	Result	q2	MVSXhc3	Wu	Result
PPC		PPC	tube	20.50	21.98	17.21	Positive	18.39	20.76	-	Positive	18.52	18.85	16.84	Positive
NPC		NPC	tube	-	-	23.27	Negative	-	-	31.92	Negative	-	-	22.23	Negative
NTC		NTC	tube	-	-	32.50	OK	-	-	-	OK	-	-	32.78	OK
1	XCA	X.spp	A1	-	-	16.36	Negative	-	-	19.31	Negative	-	-	15.51	Negative
2	XCC	X.spp	B1	-	-	18.53	Negative	-	-	23.02	Negative	-	-	17.20	Negative
3	XCVIT	X.spp	C1	-	-	18.90	Negative	-	-	24.07	Negative	-	-	17.98	Negative
4	LAL 1	LAL	D1	-	-	17.15	Negative	-	-	19.41	Negative	-	-	16.34	Negative
5	XHC 25G2	Xhc	E1	21.13	22.27	18.61	Positive	18.38	20.70	-	Positive	18.83	19.19	17.98	Positive
6	XHC 25G5	Xhc	F1	21.17	22.38	18.67	Positive	18.39	20.79	-	Positive	18.92	19.09	17.87	Positive
7	XHC 18E3	Xhc	G1	21.88	22.99	19.30	Positive	19.09	21.43	-	Positive	19.86	19.87	18.65	Positive
8	XHC 12C1	Xhc	H1	22.26	23.35	19.99	Positive	19.80	22.10	-	Positive	20.36	20.31	19.30	Positive
9	LAL 9	LAL	A2	-	-	18.87	Negative	-	-	24.42	Negative	-	-	17.78	Negative
10	LAL 3	LAL	B2	-	-	19.62	Negative	-	-	27.02	Negative	-	-	18.70	Negative
11	LAL 4	LAL	C2	-	-	19.39	Negative	-	-	25.98	Negative	-	-	18.65	Negative
12	LAL 5	LAL	D2	-	-	19.85	Negative	-	-	26.06	Negative	-	-	18.99	Negative
13	XHC 62E6	Xhc	E2	20.61	21.85	18.08	Positive	17.94	20.39	-	Positive	18.49	18.70	17.65	Positive
14	XHC 63H9	Xhc	F2	20.84	22.06	18.31	Positive	18.19	20.47	-	Positive	18.57	18.74	17.58	Positive
15	LAL 6	LAL	G2	-	-	18.54	Negative	-	-	21.89	Negative	-	-	17.75	Negative
16	LAL 13	LAL	H2	-	-	17.58	Negative	-	-	20.09	Negative	-	-	17.62	Negative
17	XHC 9A5	Xhc	A3	29.77	30.98	27.03	Positive	19.99	22.02	-	Positive	19.92	19.92	18.79	Positive
18	XHC 31E5	Xhc	B3	21.97	23.22	19.47	Positive	19.00	21.09	-	Positive	18.94	19.10	18.04	Positive
19	LAL 18	LAL	C3	-	-	17.92	Negative	-	-	23.86	Negative	-	-	17.40	Negative
20	LAL 6D2	LAL	D3	-	-	20.06	Negative	-	-	21.65	Negative	-	-	19.20	Negative
21	XHC41F8	Xhc	E3	21.17	22.56	17.98	Positive	18.93	21.20	-	Positive	18.60	19.07	17.16	Positive
22	XHC 51B2	Xhc	F3	21.10	22.33	18.55	Positive	18.52	20.81	-	Positive	18.82	19.06	17.97	Positive
23	XHC 8A3	Xhc	G3	21.12	22.37	18.53	Positive	18.72	21.02	-	Positive	18.87	19.12	17.95	Positive
24	XHC 62F2	Xhc	H3	20.40	21.64	17.93	Positive	17.49	19.98	-	Positive	18.19	18.43	17.24	Positive
25	LAL 14D8	LAL	A4	-	-	17.19	Negative	-	-	20.90	Negative	-	-	16.40	Negative

Sample code	Isolate code	expected isolate	PT	lab 6				lab 7				lab 8			
				q2	MVSXhc3	Wu	Result	q2	MVSXhc3	Wu	Result	q2	MVSXhc3	Wu	Result
26	LAL 8D2	LAL	B4	-	-	18.50	Negative	-	-	22.93	Negative	-	-	17.95	Negative
27	LAL XCVIT 5C4	LAL	C4	-	-	16.96	Negative	-	-	22.50	Negative	-	-	16.18	Negative
28	XHC 62C4	Xhc	D4	20.51	21.88	17.99	Positive	18.11	20.42	-	Positive	18.52	18.79	17.73	Positive
29	XHC 63H8	Xhc	E4	20.86	22.15	18.41	Positive	18.26	20.67	-	Positive	18.34	18.67	17.76	Positive
30	XHC 9A5*	Xhc	F4	20.75	22.08	18.31	Positive	18.06	20.57	-	Positive	18.39	18.71	17.73	Positive
31	XCA	X.spp	G4	-	-	16.12	Negative	-	-	17.48	Negative	-	-	15.51	Negative
32	XCC	X.spp	H4	-	-	18.29	Negative	-	-	20.40	Negative	-	-	17.21	Negative
33	XCVIT	X.spp	A5	-	-	33.20	Invalid	-	-	23.53	Negative	-	-	17.74	Negative
34	LAL 1	LAL	B5	-	-	17.01	Negative	-	-	21.03	Negative	-	-	16.17	Negative
35	XHC 25G2	Xhc	C5	21.08	22.43	18.38	Positive	18.68	20.96	-	Positive	18.55	18.91	17.64	Positive
36	XHC 25G5	Xhc	D5	21.25	22.68	18.60	Positive	18.24	20.83	-	Positive	18.49	18.86	17.72	Positive
37	XHC 18E3	Xhc	E5	21.96	23.34	19.22	Positive	19.46	21.86	-	Positive	19.42	19.76	18.68	Positive
38	XHC 12C1	Xhc	F5	22.42	23.73	19.92	Positive	20.00	22.18	-	Positive	19.84	20.05	19.15	Positive
39	LAL 9	LAL	G5	-	-	18.87	Negative	-	-	24.23	Negative	-	-	18.15	Negative
40	LAL 3	LAL	H5	-	-	19.71	Negative	-	-	24.10	Negative	-	-	18.67	Negative
41	LAL 4	LAL	A6	-	-	19.35	Negative	-	-	23.70	Negative	-	-	18.45	Negative
42	LAL 5	LAL	B6	-	-	19.92	Negative	-	-	26.15	Negative	-	-	18.98	Negative
43	XHC 62E6	Xhc	C6	20.84	22.16	18.27	Positive	18.71	20.83	-	Positive	18.19	18.54	17.45	Positive
44	XHC 63H9	Xhc	D6	20.92	22.42	18.33	Positive	18.35	20.61	-	Positive	18.17	18.55	17.46	Positive
45	LAL 6	LAL	E6	-	-	18.82	Negative	-	-	23.66	Negative	-	-	17.34	Negative
46	LAL 13	LAL	F6	-	-	18.46	Negative	-	-	23.86	Negative	-	-	17.64	Negative
47	XHC 9A5	Xhc	G6	22.18	23.38	19.56	Positive	19.99	22.02	-	Positive	19.69	19.86	18.83	Positive
48	XHC 31E5	Xhc	H6	21.47	22.75	18.96	Positive	19.10	21.31	-	Positive	18.74	18.98	17.92	Positive
49	LAL 18	LAL	A7	-	-	18.09	Negative	-	-	21.74	Negative	-	-	17.25	Negative
50	LAL 6D2	LAL	B7	-	-	20.02	Negative	-	-	21.40	Negative	-	-	19.23	Negative

Sample code	Isolate code	expected isolate	PT	lab 9				lab 10A				lab 10B			
				q2	MVSXhc3	Wu	Result	q2	MVSXhc 3	Wu	Result	q2	MVSXhc3	Wu	Result
PPC		PPC	tube	20.07	19.87	19.65	Positive	20.24	20.48	-	Positive	20.47	21.47	23.01	Positive
NPC		NPC	tube	37.24	36.07	25.25	Negative	-	-	22.19	Negative	-	-	23.78	Negative
NTC		NTC	tube	-	35.81	36.88	OK	-	-	-	OK	-	-	-	OK
1	XCA	X.spp	A1	36.94	36.47	17.49	Negative	-	-	22.19	Negative	-	-	21.21	Negative
2	XCC	X.spp	B1	36.52	36.03	20.14	Negative	-	-	25.82	Negative	-	-	23.54	Negative
3	XCVIT	X.spp	C1	38.58	36.32	20.61	Negative	-	-	26.32	Negative	-	-	24.09	Negative
4	LAL 1	LAL	D1	38.92	37.49	19.02	Negative	-	-	25.07	Negative	-	-	22.55	Negative
5	XHC 25G2	Xhc	E1	20.06	19.77	20.32	Positive	19.31	19.70	26.99	Positive	20.17	20.69	24.57	Positive
6	XHC 25G5	Xhc	F1	20.25	19.89	20.47	Positive	19.32	19.75	30.70	Positive	20.13	20.63	24.33	Positive
7	XHC 18E3	Xhc	G1	21.03	20.59	21.12	Positive	20.24	20.48	-	Positive	21.00	21.47	25.05	Positive
8	XHC 12C1	Xhc	H1	21.56	21.24	21.78	Positive	20.63	20.87	-	Positive	21.38	21.97	26.62	Positive
9	LAL 9	LAL	A2	37.31	35.07	20.25	Negative	-	-	25.92	Negative	-	-	24.09	Negative
10	LAL 3	LAL	B2	36.10	36.10	21.05	Negative	-	-	27.03	Negative	-	-	25.05	Negative
11	LAL 4	LAL	C2	36.53	36.05	21.20	Negative	-	-	26.60	Negative	-	-	25.00	Negative
12	LAL 5	LAL	D2	-	36.12	21.43	Negative	-	-	27.26	Negative	-	-	25.39	Negative
13	XHC 62E6	Xhc	E2	19.43	19.40	19.91	Positive	19.16	19.58	-	Positive	19.77	20.28	23.96	Positive
14	XHC 63H9	Xhc	F2	19.81	19.47	20.20	Positive	19.18	19.57	-	Positive	19.94	20.40	23.51	Positive
15	LAL 6	LAL	G2	-	36.28	20.16	Negative	-	-	26.03	Negative	-	-	23.89	Negative
16	LAL 13	LAL	H2	-	37.33	20.05	Negative	-	-	26.11	Negative	-	-	38.33	INVALID
17	XHC 9A5	Xhc	A3	21.25	20.64	21.29	Positive	20.59	20.71	38.09	Positive	21.29	21.62	25.01	Positive

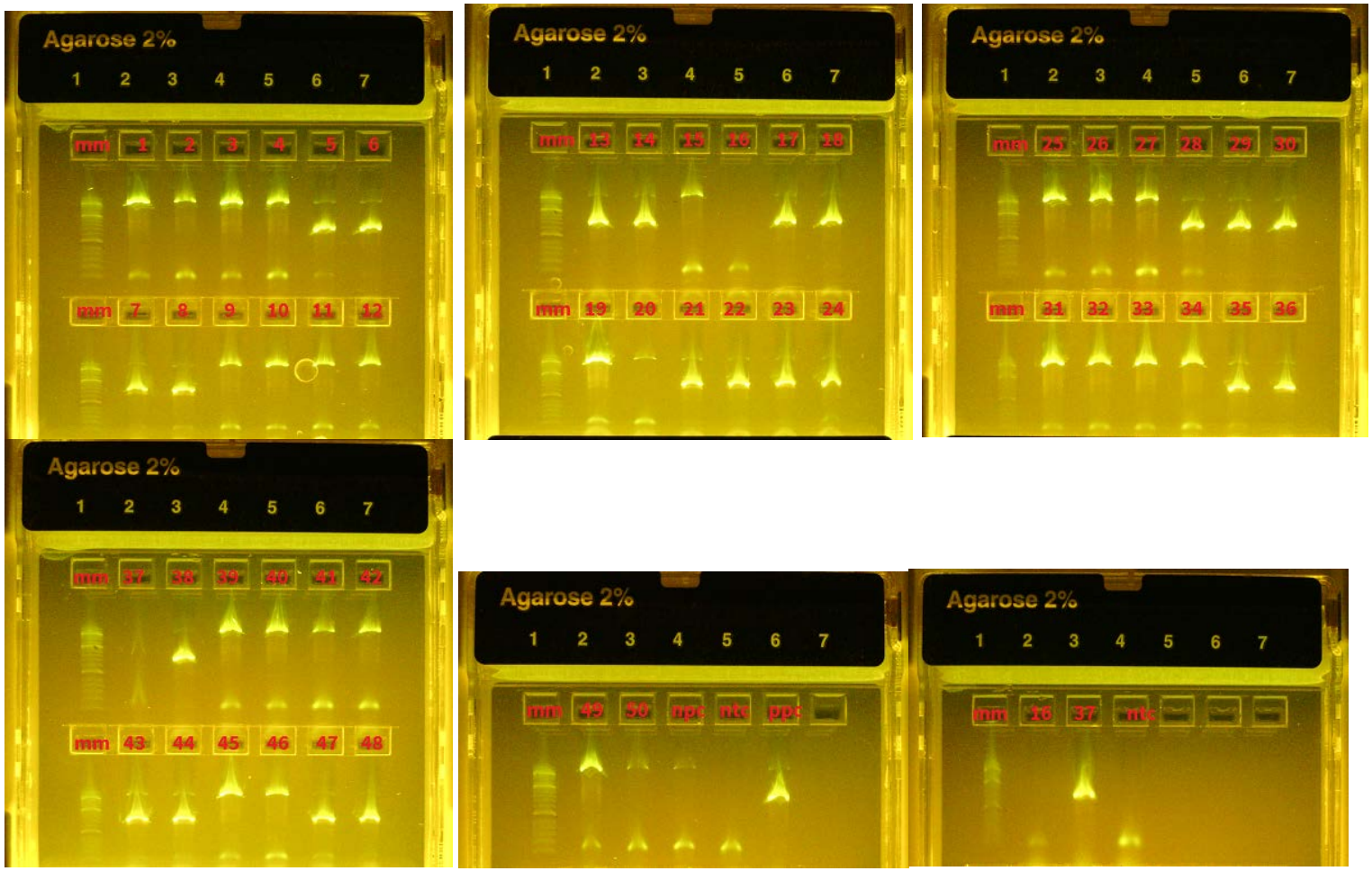
Sample code	Isolate code	expected isolate	PT	lab 9				lab 10A				lab 10B			
				q2	MVSXhc3	Wu	Result	q2	MVSXhc 3	Wu	Result	q2	MVSXhc3	Wu	Result
18	XHC 31E5	Xhc	B3	20.49	20.16	20.61	Positive	19.57	19.96	-	Positive	20.39	20.99	24.84	Positive
19	LAL 18	LAL	C3	37.18	36.05	19.57	Negative	-	-	25.14	Negative	-	-	23.19	Negative
20	LAL 6D2	LAL	D3	37.60	34.61	19.83	Negative	-	-	21.51	Negative	-	-	20.44	Negative
21	XHC41F8	Xhc	E3	20.02	19.78	19.31	Positive	19.30	19.83	27.90	Positive	20.10	20.83	22.26	Positive
22	XHC 51B2	Xhc	F3	20.19	20.03	20.77	Positive	19.42	19.76	22.23	Positive	20.29	20.89	23.74	Positive
23	XHC 8A3	Xhc	G3	20.26	19.88	20.66	Positive	19.55	19.81	-	Positive	20.38	21.03	23.91	Positive
24	XHC 62F2	Xhc	H3	19.55	19.36	20.53	Positive	18.72	19.00	-	Positive	19.56	20.18	23.81	Positive
25	LAL 14D8	LAL	A4	-	36.12	18.71	Negative	-	-	25.05	Negative	-	-	23.01	Negative
26	LAL 8D2	LAL	B4	-	-	19.07	Negative	-	-	21.06	Negative	-	-	20.13	Negative
27	LAL XCVIT 5C4	LAL	C4	37.67	34.50	18.15	Negative	-	-	22.71	Negative	-	-	21.49	Negative
28	XHC 62C4	Xhc	D4	19.37	19.32	19.56	Positive	19.06	19.35	36.41	Positive	20.04	20.40	24.01	Positive
29	XHC 63H8	Xhc	E4	19.48	19.41	19.85	Positive	19.12	19.55	35.56	Positive	19.98	20.42	24.18	Positive
30	XHC 9A5*	Xhc	F4	19.47	19.37	19.63	Positive	19.14	19.50	27.77	Positive	19.78	20.33	23.63	Positive
31	XCA	X.spp	G4	38.09	36.09	17.69	Negative	-	-	22.70	Negative	-	-	21.16	Negative
32	XCC	X.spp	H4	35.79	35.21	19.30	Negative	-	-	25.33	Negative	-	-	23.98	Negative
33	XCVIT	X.spp	A5	37.11	-	20.09	Negative	-	-	26.05	Negative	-	-	24.40	Negative
34	LAL 1	LAL	B5	39.74	37.02	18.77	Negative	-	-	24.48	Negative	-	-	23.00	Negative
35	XHC 25G2	Xhc	C5	19.89	19.71	20.01	Positive	19.16	19.57	39.53	Positive	20.10	20.51	23.91	Positive
36	XHC 25G5	Xhc	D5	20.08	19.88	20.25	Positive	19.30	19.64	-	Positive	20.24	20.74	24.31	Positive
37	XHC 18E3	Xhc	E5	20.77	20.46	20.81	Positive	20.18	20.50	29.71	Positive	21.15	21.60	24.91	Positive
38	XHC 12C1	Xhc	F5	20.26	20.20	20.49	Positive	20.71	20.91	-	Positive	21.59	22.16	24.60	Positive

Sample code	Isolate code	expected isolate	PT	lab 9				lab 10A				lab 10B			
				q2	MVSXhc3	Wu	Result	q2	MVSXhc 3	Wu	Result	q2	MVSXhc3	Wu	Result
39	LAL 9	LAL	G5	37.31	36.35	20.30	Negative	-	-	26.20	Negative	-	-	24.48	Negative
40	LAL 3	LAL	H5	38.76	34.99	20.86	Negative	-	-	26.94	Negative	-	-	25.23	Negative
41	LAL 4	LAL	A6	-	35.46	20.75	Negative	-	-	27.13	Negative	-	-	25.00	Negative
42	LAL 5	LAL	B6	35.60	36.26	21.46	Negative	-	-	27.28	Negative	-	-	25.38	Negative
43	XHC 62E6	Xhc	C6	19.70	19.55	19.99	Positive	19.02	19.43	-	Positive	19.77	20.27	23.62	Positive
44	XHC 63H9	Xhc	D6	19.73	19.57	20.12	Positive	19.20	19.57	-	Positive	20.00	20.42	24.11	Positive
45	LAL 6	LAL	E6	-	-	20.00	Negative	-	-	25.65	Negative	-	-	24.16	Negative
46	LAL 13	LAL	F6	37.03	36.23	20.14	Negative	-	-	25.30	Negative	-	-	23.79	Negative
47	XHC 9A5	Xhc	G6	21.22	20.72	21.31	Positive	20.40	20.62	37.96	Positive	21.43	21.89	25.00	Positive
48	XHC 31E5	Xhc	H6	20.25	20.27	20.83	Positive	19.57	20.09	-	Positive	20.70	21.28	-	Positive
49	LAL 18	LAL	A7	-	36.38	19.45	Negative	-	-	25.23	Negative	-	-	23.34	Negative
50	LAL 6D2	LAL	B7	39.06	37.94	19.90	Negative	-	-	21.60	Negative	-	39.97	20.37	Negative

Sample code	Isolate code	expected isolate	PT	Gel PCR			lab 11			
				Xcccar	Upbac	concl	q2	MVSXhc 3	Wu	concl
PPC		PPC	tube	+	+	Positive	20.96	21.18	18.53	Positive
NPC		NPC	tube	-	+	Negative	-	-	24.30	Negative
NTC		NTC	tube	-	-	Negative	-	-	28.28	OK
1	XCA	X.spp	A1	-	+	Negative	-	-	17.37	Negative
2	XCC	X.spp	B1	-	+	Negative	-	-	18.82	Negative
3	XCVIT	X.spp	C1	-	+	Negative	-	-	19.76	Negative
4	LAL 1	LAL	D1	-	+	Negative	-	-	18.02	Negative
5	XHC 25G2	Xhc	E1	+	+	Positive	21.18	21.24	19.40	Positive
6	XHC 25G5	Xhc	F1	+	+	Positive	21.30	21.35	19.51	Positive
7	XHC 18E3	Xhc	G1	+	+	Positive	21.96	22.08	20.28	Positive
8	XHC 12C1	Xhc	H1	+	+	Positive	22.79	22.60	21.02	Positive
9	LAL 9	LAL	A2	-	+	Negative	-	-	20.03	Negative
10	LAL 3	LAL	B2	-	+	Negative	-	-	20.41	Negative
11	LAL 4	LAL	C2	-	+	Negative	-	-	20.41	Negative
12	LAL 5	LAL	D2	-	+	Negative	-	-	21.11	Negative
13	XHC 62E6	Xhc	E2	+	+	Positive	20.69	20.93	19.08	Positive
14	XHC 63H9	Xhc	F2	+	+	Positive	21.01	21.20	19.38	Positive
15	LAL 6	LAL	G2	-	+	Negative	-	-	19.39	Negative
16	LAL 13	LAL	H2	-	-	INVALID	-	-	19.50	Negative
17	XHC 9A5	Xhc	A3	+	+	Positive	22.37	22.38	20.62	Positive
18	XHC 31E5	Xhc	B3	+	+	Positive	21.39	21.53	19.78	Positive
19	LAL 18	LAL	C3	-	+	Negative	-	-	18.98	Negative
20	LAL 6D2	LAL	D3	-	+	Negative	-	-	20.00	Negative
21	XHC41F8	Xhc	E3	+	+	Positive	21.23	21.36	18.80	Positive
22	XHC 51B2	Xhc	F3	+	+	Positive	21.38	21.57	19.70	Positive
23	XHC 8A3	Xhc	G3	+	+	Positive	21.19	21.39	19.62	Positive
24	XHC 62F2	Xhc	H3	+	+	Positive	20.69	20.76	18.94	Positive

Sample code	Isolate code	expected isolate	PT	Gel PCR			lab 11			
				Xcccar	Upbac	concl	q2	MVSXhc 3	Wu	concl
25	LAL 14D8	LAL	A4	-	+	Negative	-	-	18.26	Negative
26	LAL 8D2	LAL	B4	-	+	Negative	-	-	19.03	Negative
27	LAL XCVIT 5C4	LAL	C4	-	+	Negative	-	-	18.10	Negative
28	XHC 62C4	Xhc	D4	+	+	Positive	20.77	21.05	19.26	Positive
29	XHC 63H8	Xhc	E4	+	+	Positive	20.86	21.12	19.23	Positive
30	XHC 9A5*	Xhc	F4	+	+	Positive	20.93	21.09	19.25	Positive
31	XCA	X.spp	G4	-	+	Negative	-	-	17.33	Negative
32	XCC	X.spp	H4	-	+	Negative	-	-	18.71	Negative
33	XCVIT	X.spp	A5	-	+	Negative	-	-	19.84	Negative
34	LAL 1	LAL	B5	-	+	Negative	-	-	17.95	Negative
35	XHC 25G2	Xhc	C5	+	+	Positive	21.19	21.33	19.43	Positive
36	XHC 25G5	Xhc	D5	+	+	Positive	21.26	21.48	19.59	Positive
37	XHC 18E3	Xhc	E5	+	-	Positive	22.02	22.18	20.34	Positive
38	XHC 12C1	Xhc	F5	+	+	Positive	22.61	22.69	20.99	Positive
39	LAL 9	LAL	G5	-	+	Negative	-	-	19.87	Negative
40	LAL 3	LAL	H5	-	+	Negative	-	-	20.56	Negative
41	LAL 4	LAL	A6	-	+	Negative	-	-	20.49	Negative
42	LAL 5	LAL	B6	-	+	Negative	-	-	20.88	Negative
43	XHC 62E6	Xhc	C6	+	+	Positive	20.67	20.90	19.21	Positive
44	XHC 63H9	Xhc	D6	+	+	Positive	21.00	21.17	19.30	Positive
45	LAL 6	LAL	E6	-	+	Negative	-	-	19.43	Negative
46	LAL 13	LAL	F6	-	+	Negative	-	-	19.39	Negative
47	XHC 9A5	Xhc	G6	+	+	Positive	22.25	22.25	20.41	Positive
48	XHC 31E5	Xhc	H6	+	+	Positive	21.45	21.50	19.88	Positive
49	LAL 18	LAL	A7	-	+	Negative	-	-	19.14	Negative
50	LAL 6D2	LAL	B7	-	+	Negative	-	-	20.19	Negative

Appendix 6.4 Xhc gel based traditional method (Note: Results obtained by lab 10C for the gel-based PCR can be seen in Appendix 6.3)



Appendix 6.5 Difference in the Cq values obtained in duplicate runs of the assay

Primer set	isolates	Delta Cq in duplicate runs												
		1	2A	2B	3	4	5	6	7	8	9	10A	10B	11
Wu (based on LAL)	LAL 1	0.03	0.40	0.20	0.20	0.20	0.54	0.15	1.62	0.17	0.25	0.59	0.45	0.07
	LAL 13	0.00	0.51	0.18	0.26	0.00	0.02	0.88	3.77	0.03	0.09	0.81	INVALID	0.11
	LAL 18	0.79	0.28	0.84	0.04	0.10	0.49	0.17	2.12	0.14	0.12	0.09	0.15	0.16
	LAL 3	0.34	0.12	0.60	0.06	0.10	0.09	0.09	2.92	0.03	0.19	0.09	0.18	0.15
	LAL 4	0.16	0.15	0.58	0.04	0.20	0.06	0.04	2.28	0.21	0.45	0.53	0.00	0.08
	LAL 5	0.06	0.99	1.36	0.15	0.70	0.18	0.08	0.09	0.01	0.03	0.02	0.01	0.23
	LAL 6	0.12	0.02	0.12	0.07	0.20	0.17	0.28	1.77	0.41	0.16	0.38	0.27	0.04
	LAL 6D2	0.48	0.84	2.71	0.00	0.20	0.51	0.04	0.25	0.03	0.07	0.09	0.07	0.19
	LAL 9	0.16	0.42	0.22	0.29	0.00	0.75	0.00	0.19	0.37	0.05	0.28	0.39	0.16
	XCA	0.47	1.88	0.43	0.35	0.30	0.01	0.24	1.83	0.00	0.20	0.51	0.05	0.04
	XCC	0.33	1.53	0.42	0.20	0.10	0.21	0.24	2.62	0.01	0.84	0.49	0.44	0.23
	XCVIT	0.12	0.32	0.51	0.28	0.30	0.06	INVALID	0.54	0.24	0.52	0.27	0.31	0.08
q2 (based on Xhc)	Xhc 12C1	0.39	0.22	0.04	0.08	0.30	1.03	0.16	0.20	0.52	1.30	0.08	0.21	0.18
	Xhc 18E3	0.13	0.21	0.27	0.00	0.10	0.18	0.09	0.37	0.45	0.26	0.06	0.15	0.06
	Xhc 25G2	0.06	0.24	0.48	0.12	0.20	0.18	0.04	0.30	0.28	0.17	0.15	0.07	0.01
	Xhc 25G5	0.08	0.14	0.25	0.04	0.20	0.23	0.08	0.15	0.43	0.17	0.02	0.11	0.04
	Xhc 31E5	0.02	0.04	0.15	0.05	0.10	0.39	0.50	0.10	0.20	0.24	0.00	0.31	0.06
	Xhc 62E6	0.01	0.58	0.47	0.17	0.20	0.15	0.24	0.77	0.31	0.27	0.14	0.00	0.02
	Xhc 63H9	0.18	0.17	0.05	0.25	0.10	0.15	0.09	0.16	0.40	0.08	0.02	0.06	0.01
	Xhc 9A5	0.18	0.07	0.36	0.13	0.10	0.18	7.58	0.00	0,23	0.03	0.19	0.14	1.44
MVSXhc3 (based on Xhc)	Xhc 12C1	0.38	0.21	0.21	0.14	0.30	0.65	0.38	0.08	0.26	1.04	0.04	0.19	0.09
	Xhc 18E3	0.02	0.22	0.13	0.01	0.00	0.03	0.35	0.43	0.11	0.13	0.02	0.13	0.10
	Xhc 25G2	0.04	0.04	0.36	0.03	0.10	0.07	0.15	0.26	0.28	0.06	0.13	0.18	0.09
	Xhc 25G5	0.00	0.84	0.35	0.06	0.00	0.09	0.30	0.04	0.23	0.01	0.11	0.11	0.13
	Xhc 31E5	0.20	0.05	0.01	0.03	0.10	0.51	0.46	0.22	0.13	0.11	0.13	0.29	0.03
	Xhc 62E6	0.00	0.78	0.23	0.14	0.30	0.21	0.31	0.44	0.16	0.15	0.15	0.01	0.03
	Xhc 63H9	0.10	0.16	0.01	0.19	0.10	0.15	0.36	0.14	0.19	0.10	0.00	0.02	0.03
	Xhc 9A5	0.37	0.27	0.23	0.06	0.20	0.11	7.60	0.00	0.05	0.08	0.09	0.27	1.29