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Research

Genome-Informed Multiplex Conventional PCR for Identification and Differentiation of *Xanthomonas citri* pv. *citri* Subpathotypes, the Causal Agents of Asiatic Citrus Canker

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Abstract

Asiatic citrus canker, one of the most important diseases of citrus, is caused by *Xanthomonas citri* pv. *citri*. It has high economic impact and can spread easily, and the disease is difficult to manage; it is a quarantine organism in many citrus-producing countries. *X. citri* pv. *citri* has been separated into three subpathotypes (A, A^*, A^w) that differ in host range and geographical distribution, thus creating a need to differentiate subpathotypes for surveillance and disease management. Availability of useful diagnostic tools is the cornerstone of successful surveillance, quarantine, and eradication measures. In this study, a multiplex conventional PCR (cPCR) assay was developed for detection and subpathotype determination of *X. citri* pv. *citri*. Assay specificity was assessed by four different labs on a total of 146 *X. citri* pv. *citri* and 58 other *Xanthomonas* isolates. The assay demonstrated high analytical sensitivity, specificity, and selectivity. False negatives were observed with A Lineage 2 strains, and potential false positives were observed for *X. citri* pv. *bilvae*. Combined with a simple extraction protocol, the assay has been deployed successfully at the Plant Protection and Quarantine Plant Pathogen Confirmatory Diagnostics Laboratory. This assay has proven useful for differentiating Asiatic citrus canker subpathotypes from symptomatic citrus tissue.

Keywords: Asiatic citrus canker, diagnostic, differentiation, discrimination, identification, pathotype, PCR, *Xanthomonas axonopodis* pv. *citri*, *Xanthomonas citri* pv. *citri*

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Citrus bacterial canker (CBC) is one of the most economically important diseases of citrus worldwide (Gottwald et al. 2001, 2002; Graham et al. 2004). It is caused by a gram-negative γ-proteobacterium represented by two phylogenetically distinct groups: (i) *Xanthomonas citri* pv. *citri* (Doidge 1916; Dowson 1939; Gabriel et al. 1989;

Schaad et al. 2006) (synonymous with *X. axonopodis* pv. *citri* [Xac] [Vauterin et al. 1995], *X. citri* subsp. *citri* [Euzéby 2007], *X. campestris* pv. *citri* [Young et al. 1978, 1991], and *X. smithii* subsp. *citri* [Schaad et al. 2005]) that originated from Asia and (ii) *X. citri* pv. *aurantifolii* (Constantin et al. 2016) (synonymous with *X. axonopodis* pv. *aurantifolii* [Vauterin et al. 1995], *X. campestris* pv. *aurantifolii* [Young et al. 1978, 1991], and *X. fuscans* subsp. *aurantifolii* [Schaad et al. 2005]) that originated in South America. *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii* cause Asiatic (pathotype A) and South American citrus canker (pathotypes B and C), respectively, and their canker-like symptoms are visually indistinguishable on their susceptive hosts (Jaciani et al. 2012; Pruvost et al. 2015; Robène et al. 2020).

X. citri pv. *citri* most likely originated on the Indian subcontinent but now exists in more than 30 countries (Civerolo 1982, 1984, 1994; Patané et al. 2019). Similarly, the so-called ancestral species of its primary host genus (*Citrus*) originated from several Asian regions (Northeast India, China, Malay Archipelago, and Indonesia) (Ollitrault et al. 2020; Ollitrault and Navarro 2012; Talon et al. 2020; Wu et al. 2018). Asiatic citrus canker (CBC-A) strains are prevalent currently in the Indian Ocean region, East Asia, South America, and the United States (Florida and Texas) (Gottwald et al. 2001, 2002; Graham et al. 2004). Genotyping techniques have been used to evaluate genetic and pathological diversity among CBC-A strains (Al-Saadi et al. 2007; Bui Thi Ngoc et al. 2008, 2009, 2010; Cubero and Graham 2002; Escalon et al. 2013; Gordon et al. 2015; Jaciani et al. 2012; Jeong et al. 2019; Lee et al. 2008; Li et al. 2007; Pruvost et al. 2014, 2015) and have separated CBC-A into three subpathotypes (A, A^*, A^w) (Bui Thi Ngoc et al. 2009; Gordon et al. 2015; Jeong et al. 2019; Pruvost et al. 2014, 2015). Subpathotype A strains split into two clusters, forming Lineage 1 and Lineage 2 (recently reported and less studied), whereas A^w and A^* strains were deemed Lineage 3 and Lineage 4, respectively (Pruvost et al. 2014); this genetic structure was confirmed using whole-genome sequencing (WGS) data (Gordon et al. 2015). Subpathotype A is the most widely distributed and infects a broad range of hosts, including many *Citrus* species, hybrids, or related genera such as trifoliate orange (*Citrus trifoliata*) (Civerolo 1982, 1984; Ference et al. 2018; Graham et al. 2004; Stall and Civerolo 1991; Vernière et al. 1998). Subpathotype A^* strains are currently limited to Asia, the Arabian Peninsula, and East Africa and have a limited host range, with most outbreaks occurring on Mexican lime (or key lime) (*Citrus* × *aurantifolia* var. *aurantifolia*) (Derso et al. 2009; Pruvost et al. 2015; Vernière et al. 1998). Subpathotype A^w is known currently to occur in the United States (Florida and Texas), the Arabian Peninsula, and on the Indian subcontinent, with natural infections seemingly restricted to Mexican lime and alemow (*C.* × *aurantifolia* var. *macrophylla*) (Rybak et al. 2009; Sun et al. 2004). Interestingly, subpathotype A^w was first reported in Florida but was found to have likely originated from India (Bui Thi Ngoc et al. 2009; Patané et al. 2019; Schubert et al. 2001).

Compared with the *X. citri* pv. *citri* (CBC-A), *X. citri* pv. *aurantifolii* (CBC-B and CBC-C) is considered a mild pathogen of citrus (Jaciani et al. 2012). CBC-B strains cause less severe symptoms, have a restricted host range, and were known to be present in only Argentina, Paraguay, and Uruguay (Goto et al. 1980; Schubert et al. 2001; Vernière et al. 1998). CBC-B is said to no longer exist in nature, having been eradicated due to competition from the more aggressive and pathogenic CBC-A (CABI 2021; Schubert et al. 2001). CBC-C, dubbed Mexican lime canker because it only infects Mexican lime, was limited to the state of São Paulo, Brazil (Civerolo 1982, 1984, 1994; Fonseca et al. 2019a; Namekata and Oliveira 1972; Rossetti 1977; Vernière et al. 1998), but is also said to no longer exist in nature (CABI 2021).

Aside from its socioeconomic impact, citrus canker disease has implications for national and international trade. Like other xanthomonads, Asiatic citrus canker strains spread locally through wind and rain dispersion, or long distances by dissemination of infected citrus material (Graham et al. 2004). Citrus canker disease has high economic impact and is difficult to manage, and *X. citri* pv. *citri* is listed as a quarantine organism in citrus-producing countries such as Australia, New Zealand, South Africa, North Africa, countries in the European Union, and several U.S. states, where disease has been deemed eradicated or not introduced (Pruvost et al. 2014, 2015; Robène et al. 2020). Successful implementation of surveillance, quarantine measures to prevent spread and establishment in new areas, and eradication efforts depend on the availability of useful diagnostic tools (Robène et al. 2020). Numerous molecular detection assays have been developed, including conventional PCR (cPCR) (Cubero and Graham 2002; Fonseca et al. 2019b; Hartung et al. 1996; Kingsley and Fritz 2000; Miyoshi et al. 1998), real-time quantitative PCR (qPCR) (Cubero and Graham 2005; Mavrodieva et al. 2004; Robène et al. 2020), and loop-mediated isothermal amplification (LAMP) (Rigano et al. 2010) assays.

In the United States, both subpathotypes A and A^w have become established in two major commercial citrus-producing states (Florida and Texas), resulting in quarantine of affected areas and routine testing and surveillance of affected and surrounding areas. The A subpathotype has also been found in Louisiana (groves) and North Carolina (nurseries). Citrus canker outbreaks in the state of Texas represent a unique situation. Commercial citrus production is primarily located in the Lower Rio Grande Valley, where the main commercial citrus crop is grapefruit (*C. paradisi*). Subpathotype A^w has only been found on lime in residential settings in this area of Texas (da Graça et al. 2017), whereas subpathotype A has only been found in the Greater Houston area (Perez et al. 2021). A portion of both the Lower Rio Grande Valley and Greater Houston area represent distinct quarantine areas. Quarantine zones are not connected, and material within each area is regulated to prevent movement and further introduction of the pathogen to new areas. It is important to determine infecting *X. citri* pv. *citri* subpathotypes in these areas to ensure the effectiveness of quarantine measures to prevent the A subpathotype from reaching commercial grapefruit-production areas, as well as to prevent the spread of the A^w subpathotype outside the Lower Rio Grande Valley region. Stakeholders and the Citrus Health Response Program (CHRP), a United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) Plant Protection and Quarantine (PPQ) domestic program, expressed a need for effective detection and differentiation of citrus canker pathogens.

Current molecular assays were designed to detect and differentiate entire groups of citrus canker isolates (CBC-A, CBC-B, or CBC-C). Additionally, follow-up pathotyping assays, such as amplified fragment length polymorphism (AFLP) (Bui Thi Ngoc et al. 2010) or multi-locus variable number of tandem repeats analysis (MLVA) (Pruvost et al. 2014), require skilled personnel and are laborious and time consuming. Only *X. citri* pv. *citri* (CBC-A) has major agricultural significance, as it is more aggressive and widespread, and it has been the only one found associated with serious canker outbreaks, even in countries where CBC-B and CBC-C have historically occurred concomitantly (Robène et al. 2020; Schubert et al. 2001). Therefore, the USDA APHIS PPQ Science & Technology (S&T) Plant Pathogen Confirmatory Diagnostics Laboratory (PPCDL) focused efforts on CBC-A discrimination and used comparative genomics to identify unique regions useful for differentiation of infecting subpathotypes. To fulfill stakeholder needs and simplify testing, unique targets were

multiplexed into a single cPCR reaction, along with a control primer set designed to detect the entire CBC-A group, which was coupled downstream of a simple sample processing method. This report describes the development and validation of a multiplex cPCR assay for detection and subpathotype determination of CBC-A on citrus.

MATERIALS AND METHODS

Bacterial isolates and DNA extraction

One hundred *Xanthomonas* isolates were used in this study for initial analytical specificity and selectivity testing, including 94 *X. citri* pv. *citri*, two *X. citri* pv. *aurantifolii*, and four *X. euvesicatoria* pv. *citrumelonis* (Table 1); an additional 104 unique isolates were used by laboratories participating in ring-test validation (Supplementary Tables S1 to S3). Isolates were selected to include A, A^w, and A^* subpathotypes and represent a broad host range and diverse spatial and temporal distribution (analytical specificity). Bacterial DNA was provided, or bacterial cultures were grown in Nutrient Broth (MilliporeSigma, St. Louis, MO, U.S.A.) at 30°C with shaking for 24 h. DNA was isolated from bacterial cells using the *Quick*-DNA Fungal/Bacterial Midiprep Kit (Zymo Research, Irvine, CA, U.S.A.) and then quantified using the Qubit 3.0 Fluorometer (Thermo Fisher Scientific Life Technologies, Waltham, MA, U.S.A.), and working solutions were made at 1 ng/µl.

Primer design

The complete genome sequences (chromosome) for *X. axonopodis* pv. *citri* str. 306 (A subpathotype) and *X. citri* subsp. *citri* Aw12879 (A^w subpathotype), as well as a contig (J058_Contig001.1) of *X. citri* pv. *citri* strain AS270 (A^* subpathotype), were downloaded from the National Center for Biotechnology Information (NCBI) GenBank; accession numbers were AE008923.1, CP003778.1, and JPLN01000001, respectively. Sequences were aligned with progressiveMauve v2.4.0 (Darling et al. 2010), and unique regions were identified for each subpathotype. Primers (Table 2) were designed using Geneious v10.0 (Kearse et al. 2012) and evaluated in silico using NCBI Primer BLAST (Altschul et al. 1990) to assess matches with nontargets (specificity). Primer binding sites for set aF5/aR5 are located within a hypothetical protein. Binding sites for primers StarF5 and StarR5 are located within a CcdA and CcdB family protein, respectively, as part of a type II toxin-antitoxin system. Binding sites for primers WF3 and WR3 are located within a hypothetical protein and IS4 family transposase, respectively. The significance of these unique target sites has not been evaluated, aside from their subpathotype-specific nature.

cPCR reaction conditions

The primer sets developed in this study were tested along with a control primer set (Table 2) designed to detect all Asiatic citrus canker subpathotypes (Park et al. 2006). Individual primer sets were initially screened in reactions containing 1X PCR buffer, 0.2 mM dNTPs, 3 mM $MgCl_2$, 2 U Platinum *Taq* DNA Polymerase (Thermo Fisher Scientific), 200 nM of each primer (Table 2), and 2 µl of DNA template in a final reaction volume of 25 µl. Multiplex cPCR reactions contained 12.5 µl of 2X QIAGEN Multiplex PCR Master Mix and 5 µl of 5X Q Solution, as part of the QIAGEN Multiplex PCR Kit (Qiagen, Germantown, MD, U.S.A.), 200 nM of each primer (Table 2), and 1 µl of DNA template in a final reaction volume of 25 µl. For diagnostic application, the DNA template input was increased by 2 µl to reduce potential pipetting error. All reactions were run in the ProFlex PCR System (Applied Biosystems, Waltham, MA, U.S.A.), or equivalent thermocycler, according to the following cycling conditions: initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 63°C for 90 s, and elongation at 72°C for 1 min, and then a final extension at 72°C for 10 min. Samples were held at 4°C in the thermocycler until downstream analysis was performed.

Gel electrophoresis

PCR products were analyzed using 1.5% agarose gels prepared using 1X TAE buffer. For analysis, 8 µl of PCR product was mixed with 2 µl of 6X BioTracker loading buffer (BioVentures, Murfreesboro, TN, U.S.A.); all 10 µl were added to lane wells. Samples were run alongside a 100-bp ladder (BioVentures). Gels were run in 1X TAE buffer for 1 h at 100 V using a Horizon 11.14 Gel Electrophoresis System (Biometra, Beverly, MA, U.S.A.) and PowerPac Basic Power Supply (Bio-Rad, Hercules, CA, U.S.A.), or equivalents. Gels were stained for 10 to 15 min using 0.58 µg/ml ethidium bromide (EtBr) solution (35 µl of 5 mg/ml EtBr concentrated stock solution in 300 ml of dH_2O) or 1X GelRed nucleic acid gel stain (Biotium, Fremont, CA, U.S.A.), followed by destaining for 10 to 15 min in ddH_2O; an orbital shaker set at 50 rpm was used for both stages. Gels were imaged using the G:Box Gel Documentation System and GeneSys software (Syngene, Frederick, MD, U.S.A.). Alternatively, PCR products were analyzed using the 4200 TapeStation System, D1000 ScreenTape, D1000 DNA Ladder, D1000 Sample Buffer, and 4200 TapeStation Controller software (Agilent Technologies, Santa Clara, CA, U.S.A.), according to the manufacturer's instructions.

Specificity and sensitivity testing

Each isolate was tested initially using each primer set (Table 2) individually to determine primer analytical specificity and assess cross-reactivity (selectivity). The ImmunoStrip for *Xanthomonas axonopodis* pv. *citri* (Xac) (Agdia, Elkhart, IN, U.S.A.) was used in some cases to aid in subpathotype determination. The Xac ImmunoStrip detects the A subpathotype and has known cross-reactions with *X. euvesicatoria* pv. *citrumelonis*; it does not detect A^w and A^* subpathotypes (information is available in the product manual; m251.1). Tenfold serial dilutions of DNA extracted from Xac A3213 (A), Xac 0053 (A^w), and Xac 270 (A^*), ranging from 100 pg/µl to 1 fg/µl, were used to determine assay analytical sensitivity; dilutions were made in molecular-grade water. Three fivefold serial dilutions, starting from the lowest concentration producing appropriate bands, were made and tested to determine the limit of detection. Approximate copy number per reaction was calculated based on the average size of all *X. citri* pv. *citri* genomes in NCBI (5.3 Mb) using the following formula, where X is the amount of DNA (ng) and N is the length of dsDNA (bp):

$$\text{number of copies (molecules)} = \frac{\text{X ng} * 6.0221 \times 10^{23} \frac{\text{molecules}}{\text{mole}}}{\left(\text{N} * 660 \frac{\text{g}}{\text{mole} * \text{bp}}\right) * 1 \times 10^{9} \frac{\text{ng}}{\text{g}}}$$

The analytical sensitivity of the assay when all primer sets were multiplexed into a single reaction (quadruplex) was determined using the same set of dilutions used to determine the sensitivity of each individual primer set. Multiplex assay analytical

TABLE 1

Isolates used for initial feasibility testing and primer assessment[a]

Isolate[b]	Organism[b]	Host	Country	Year	Known subpathotype	Source[c]	Xac F/R (all)	aF5/R5 (A)	WF3/R3 (Aw)	Star F5/R5 (A*)
53	Xcc	*Citrus* × *aurantifolia* var. *aurantifolia*	U.S.A., FL		A[w]		+	−	+	−
205	Xcc	*Citrus* × *aurantifolia* var. *aurantifolia*			A*		+	−	−	+
270	Xcc				A*		+	−	−	+
1609	Xcc				A*	N. Wang	+	−	−	+
2032	Xcc				A[w]	N. Wang	+	−	+	−
160042	Xcc		U.S.A., TX		A[w]		+	−	+	−
160149	Xcc		U.S.A., TX		A[w]		+	−	+	−
160197	Xcc		U.S.A., TX		A[w]		+	−	+	−
B23	Xcc		Reunion		A	O. Pruvost	+	+	−	−
B	Xca				B		−	−	−	−
BRN16 (CFBP 3114)	Xec			1984	E	O. Pruvost	−	−	−	−
C	Xca				C		−	−	−	−
C40	Xcc	*Citrus sinensis*	Reunion	1988	A	O. Pruvost	+	+	−	−
CFBP 1209	Xcc	*Citrus maxima*	Hong Kong	1963	A	O. Pruvost	+	+	−	−
CFBP 2548	Xcc	*Citrus* sp.	Taiwan		A	O. Pruvost	+	+	−	−
CFBP 2851	Xcc	*Citrus* sp.	India	1948	A	O. Pruvost	+	+	−	−
CFBP 2854	Xcc	*Citrus* sp.	Japan	1958	A	O. Pruvost	+	+	−	−
CFBP 2911	Xcc	*Citrus* sp.	Pakistan	1984	A*	O. Pruvost	+	−	−	+
F1 (CFBP 3138)	Xec			1984	E	O. Pruvost	−	−	−	−
F100 (JJ238-28)	Xec			1985	E	O. Pruvost	−	−	−	−
F132 (JJ238-29)	Xcc			1986	A	O. Pruvost	+	+	−	−
F135 (JJ238-30)	Xcc			1986	A	O. Pruvost	+	+	−	−
F494 (JJ238-34)	Xcc			1988	A	O. Pruvost	+	+	−	−
F554 (JJ238-35)	Xcc			1989	A	O. Pruvost	+	+	−	−
F563 (JJ238-37)	Xcc			1989	A	O. Pruvost	+	+	−	−
F598 (JJ238-38)	Xcc			1989	A	O. Pruvost	+	+	−	−
F600 (JJ238-39)	Xcc			1989	A	O. Pruvost	+	+	−	−
F601 (JJ238-40)	Xcc			1989	A	O. Pruvost	+	+	−	−
F6 (CFBP 2910)	Xec			1984	E	O. Pruvost	−	−	−	−
JA151-8	Xcc		Reunion		A	O. Pruvost	+	+	−	−
JF090-2	Xcc	*Citrus* × *aurantifolia* var. *aurantifolia*	Oman	1986	A*	O. Pruvost	+	−	−	+
JF090-8	Xcc	*Citrus* × *aurantifolia* var. *aurantifolia*	Oman	1986	A[w]	O. Pruvost	+	−	+	−
JH081-2	Xcc	*Citrus* sp.	China	1988	A	O. Pruvost	+	+	−	−
JJ001-3	Xcc				A		+	+	−	−
JJ036-2	Xcc	*Citrus maxima*	Thailand	1989	A	O. Pruvost	+	+	−	−
JJ201-2	Xcc	*Citrus* sp.	Japan		A	O. Pruvost	+	+	−	−
JJ223-2	Xcc	*Citrus sinensis*	Philippines		A	O. Pruvost	+	+	−	−
JJ238-10	Xcc	*Citrus* × *aurantifolia* var. *aurantifolia*	Maldives	1987	A	O. Pruvost	+	+	−	−
JJ238-24	Xcc	*Citrus* × *aurantifolia* var. *aurantifolia*	Thailand	1989	A*	O. Pruvost	+	−	−	+
JK002-10	Xcc	*Citrus* × *aurantifolia* var. *aurantifolia*	Saudi Arabia	1988	A*	O. Pruvost	+	−	−	+
JK004-1	Xcc	*Citrus* sp.	China	<1990	A	O. Pruvost	+	+	−	−
JK146-4	Xcc	*Citrus trifoliata*	Malaysia	1990	A	O. Pruvost	+	+	−	−
JK163-1	Xcc		Mauritius		A	O. Pruvost	+	+	−	−
JM035-2	Xcc	*Citrus* × *aurantifolia* var. *aurantifolia*	Saudi Arabia	<1993	A*	O. Pruvost	+	−	−	+
LG097	Xcc	*Citrus limon*	Bangladesh	2006	A (Lineage 2)	O. Pruvost	+	−	−	−
LG098	Xcc	*Citrus* × *aurantifolia* var. *aurantifolia*	Bangladesh	2006	A	O. Pruvost	+	+	−	−
LG115	Xcc	*Citrus* sp.	India	2007	A[w]	O. Pruvost	+	−	+	−
NCPPB 3562	Xcc	*Citrus limon*	India	1988	A (Lineage 2)	NCPPB	+	−	−	−
NCPPB 3607	Xcc	*Citrus* × *aurantifolia* var. *aurantifolia*	India	1988	A*	NCPPB	+	−	−	+
NCPPB 3608	Xcc	*Citrus* × *aurantifolia* var. *aurantifolia*	India	1988	A[w]	NCPPB	+	−	+	−

(*Continued on next page*)

[a] Blank cells represent isolate information that was not provided or is otherwise unavailable or unknown.

[b] Xcc = *Xanthomonas citri* pv. *citri*; Xca = *Xanthomonas citri* pv. *aurantifolii*; and Xec = *Xanthomonas euvesicatoria* pv. *citrumelonis*.

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specificity was evaluated further during assay validation testing (see corresponding section). Tests were also performed to determine assay functionality under hypothetical mixed-infection conditions. High (0.1 ng/µl) or low (100 fg/µl) concentrations of Xac A3213 (A), Xac 0053 (A^w), and Xac 270 (A^*) DNA were mixed in different combinations, according to a 3 × 2 matrix, to determine any effects caused by the presence of multiple infecting subpathotypes and their relative titers.

PCR product sequencing

PCR amplicons from Xac A3213 (A), Xac 0053 (A^w), and Xac 270 (A^*) were subjected to Sanger sequencing. Briefly, PCR products were cleaned using ExoSAP-IT *Express* PCR Product Cleanup (Thermo Fisher Scientific Life Technologies), according to the manufacturer's instructions. Sequencing reactions were carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific Life Technologies), according to the manufacturer's instructions. Sequencing reactions were purified using the BigDye Xterminator Purification Kit (Thermo Fisher Scientific Life Technologies), according to the manufacturer's instructions and then run in a SeqStudio Genetic Analyzer (Applied Biosystems). Sequences were queried by BLASTn against the NCBI nucleotide collection, whole-genome shotgun contigs (wgs) for *Xanthomonas*, NCBI Genomes (chromosome), and RefSeq Genome to determine sequence identity.

TABLE 1 (*Continued from previous page*)

Isolate[b]	Organism[b]	Host	Country	Year	Known subpathotype	Source[c]	Xac F/R (all)	aF5/R5 (A)	WF3/R3 (Aw)	Star F5/R5 (A*)
NCPPB 3615	Xcc	*Citrus* × *aurantifolia* var. *aurantifolia*	India	1989	A*	NCPPB	+	−	−	+
X 88	Xcc		Argentina			J. Hartung	+	+	−	−
X02 1036	Xcc		U.S.A., FL		A^w	DPI	+	−	+	−
X03 1003	Xcc		U.S.A., FL		A^w	DPI	+	−	+	−
X03 1004	Xcc		U.S.A., FL		A^w	DPI	+	−	+	−
X03 1005	Xcc		U.S.A., FL		A^w	DPI	+	−	+	−
X03 1035	Xcc		U.S.A., FL		A^w	DPI	+	−	+	−
X03 11-866	Xcc		U.S.A., FL		A	DPI	+	+	−	−
X03 1221	Xcc		U.S.A., FL		A	DPI	+	+	−	−
X2002-1035p	Xcc			2002	A^w	O. Pruvost	+	−	+	−
X2003-01029g	Xcc	*Citrus* sp.	U.S.A., FL	2003	A^w	O. Pruvost	+	−	+	−
X2003-3218	Xcc	*Citrus* sp.	U.S.A., FL	2003	A^w	O. Pruvost	+	−	+	−
Xac A3213	Xcc				A (type strain)	O. Pruvost	+	+	−	−
Xc1	Xcc		Argentina			J. Hartung	+	+	−	−
Xc100 (LMG 9665)	Xcc	*Citrus* sp.	Pakistan	1984		J. Hartung	+	−	−	+
Xc101	Xcc		Guam			J. Hartung	+	+	−	−
Xc106	Xcc		Australia			J. Hartung	+	+	−	−
Xc111	Xcc		China			J. Hartung	+	+	−	−
Xc115 (NCPPB 409)	Xcc	*Citrus limon*	New Zealand	1957		J. Hartung	+	+	−	−
Xc118 (PDDCC24-85)	Xcc					J. Hartung	+	+	−	−
Xc126	Xcc	*Citrus reticulata*	Korea	1987		J. Hartung	+	+	−	−
Xc127	Xcc	*Citrus* × *aurantifolia* var. *aurantifolia*	Maldives	1987		J. Hartung	+	+	−	−
Xc138	Xcc		Philippines	1988		J. Hartung	+	+	−	−
Xc151	Xcc		India			J. Hartung	+	−	−	+
Xc158	Xcc	*Citrus sinensis*	Pakistan	1988	A	J. Hartung	+	+	−	−
Xc176 (LMG 9662)	Xcc	*Citrus* × *aurantifolia* var. *aurantifolia*	Saudi Arabia		A	J. Hartung	+	−	−	−
Xc180	Xcc		China			J. Hartung	+	+	−	−
Xc200	Xcc		Thailand			J. Hartung	+	+	−	−
Xc202	Xcc		Malaysia			J. Hartung	+	+	−	−
Xc206	Xcc		Oman			J. Hartung	+	−	−	+
Xc208	Xcc		Oman			J. Hartung	+	−	+	−
Xc211	Xcc		Hong Kong			J. Hartung	+	+	−	−
Xc225	Xcc		Brazil			J. Hartung	+	+	−	−
Xc232	Xcc		China			J. Hartung	+	+	−	−
Xc251	Xcc	*Citrus* sp.	Yemen	1988		J. Hartung	+	+	−	−
Xc255	Xcc		Thailand			J. Hartung	+	+	−	−
Xc259	Xcc		China			J. Hartung	+	−	−	+
Xc269	Xcc	*Citrus* × *aurantifolia* var. *aurantifolia*	Saudi Arabia	1988	A*	J. Hartung	+	−	−	+
Xc336	Xcc		Uruguay			J. Hartung	+	+	−	−
Xc360	Xcc		Vietnam			J. Hartung	+	+	−	−
Xc362	Xcc		Australia			J. Hartung	+	+	−	−
Xc410	Xcc		Reunion			J. Hartung	+	+	−	−
Xc447	Xcc		Micronesia			J. Hartung	+	+	−	−
Xc46	Xcc		India			J. Hartung	+	+	−	−
Xc62	Xcc	*Citrus* sp.	Japan	1978	A	J. Hartung	+	+	−	−
Xc63	Xcc		Japan	1978		J. Hartung	+	+	−	−
Xc78	Xcc		Uruguay			J. Hartung	+	+	−	−
Xc85	Xcc		Japan			J. Hartung	+	+	−	−
XCC 406G	Xcc				A*	DPI	+	−	−	+
XS2004-00010	Xcc				A^w	O. Pruvost	+	−	+	−

Assay validation testing

Three outside laboratories participated in assay validation: (i) Florida Department of Agriculture and Consumer Services, the Division of Plant Industry (DPI) (Gainesville, FL, U.S.A.); (ii) University of Florida (Gainesville, FL, U.S.A.); and (iii) CIRAD (St. Pierre, Réunion, France). Primers and positive controls were prepared, validated, and shipped to participants, along with a copy of the testing protocol. Each participating laboratory tested known isolates from their collections to further establish analytical specificity and selectivity metrics, instead of testing a blind panel of identical samples; acceptable DNA concentrations for testing ranged from 0.1 to 0.01 ng/µl. The numbers and types of samples tested are shown in Table 3. Certain samples were also evaluated with a real-time PCR assay developed and used by the PPCDL (Santillana et al. 2021) to determine relative titers and compare results with the assay described in this report.

Plant sample preparation

Leaf samples exhibiting citrus canker symptoms were received by the PPCDL for testing and processed by a bacterial streaming method; this method is specific for symptomatic tissue and was used for time- and cost-saving purposes. Briefly, an approximately 3 × 3-mm piece of water-soaked tissue around a necrotic lesion was excised using a new disposable razor blade; one lesion each from three individual leaves were used and pooled into a composite sample. The three excised pieces were added to a microfuge tube containing 200 µl of TE buffer (pH 8.0) and then incubated for 10 min at room temperature with shaking at 300 rpm using a thermomixer. After "streaming," 100 µl of suspension was transferred to a separate microfuge tube containing 100 µl of 20% Chelex 100 Chelating Resin (Bio-Rad) in TE buffer. Samples were thoroughly mixed, incubated for 5 min at 95°C in a thermomixer, and then snap-chilled on ice for 5 min. Sample tubes were then centrifuged at 8,000 × *g* for 5 min. Supernatant was removed to a clean microfuge tube and then kept on ice for immediate use or stored at −20°C for future use.

The original protocol used during initial testing was as follows: (i) excised tissue was incubated in water instead of TE buffer; (ii) 100 µl of suspension was transferred to a clean microfuge tube and then boiled at 99°C for 10 min instead of 95°C for 5 min. All other aspects of the process were the same. The original process worked for most of the samples; however, carryover of inhibitory substances, such as endogenous plant molecules or exogenous compounds from foliar sprays, produced erroneous cPCR results (lack of bands) for some samples. Leaf samples were tested by both the real-time and cPCR assays.

RESULTS

Primer specificity

Specificity was checked initially for each primer set individually. All *X. citri* pv. *citri* isolates (Table 1) tested positive with the Xac primer set (Park et al. 2006), which was designed to detect all Asiatic citric canker subpathotypes (A, A^w, and A^*), as determined by the presence of a 561-bp band (gel data not shown). Isolates of *X. citri* pv. *aurantifolii* (B and C subpathotypes) and *X. euvesicatoria* pv. *citrumelonis* did not react with this primer set. All A^w and A^* isolates tested (Table 1) produced bands only with their respective subpathotype-specific primer set, as evident by the presence of a 346-bp and 143-bp band (gel data not shown), respectively. Primers designed to detect subpathotype A strains produced the appropriate 455-bp band for all A isolates tested (Table 1), with few exceptions. Isolates NCPPB 3562 and LG097 (A isolates of Lineage 2), as well as Xc176, failed to produce the 455-bp subpathotype A band, only producing the 561-bp general Xac control band. It is unknown if Xc176 was Lineage 2 or some other type of variant. When tested with the Xac ImmunoStrip, which detects A subpathotypes (does not detect A^w and A^*), NCPPB 3562 produced negative results, corroborating the atypical results observed with the multiplex cPCR. PCR amplicons for the representative strains of A, A^w, and A^* were sequenced and subjected to BLAST analysis. Amplicons for each primer set matched with 100% identity to isolates of their target subpathotype within the GenBank database, confirming assay specificity.

Analytical sensitivity

Sensitivity was checked initially for each primer set individually, then tested again in multiplex format. Band intensity for all primer sets fell below detectable levels at input concentrations lower than 100 fg/µl (Supplementary Fig. S1). At this concentration, the calculated copy number per reaction based on a 1-µl input was approximately 17. At 100 fg/µl (17 copies/reaction), the percent positive for the Xac-, A-, A^w-, and A^*-specific primer sets was 77.8, 83.3, 94.4, and 72.2%, respectively, based on results from six replicate wells tested three times ($N = 18$). At an input concentration of 200 fg/µl, all primer sets were

TABLE 3

Numbers and types of samples tested by participant laboratories

Laboratory	A	A^w	A^*	Other	Total
DPI	16	4	2	6	28
University of Florida	1	1	4	1	7
CIRAD	21	7	16	47	91
Total	38	12	22	54	126

TABLE 2

Oligonucleotide primers used in this study

Primer	Sequence (5′→3′)	Subpathotype target	Size (bp)	Reference
Xac F	CGTCGCAATACGATTGGAAC	Xac (All)	561	Park et al. 2006
Xac R	CGGAGGCATTGTCGAAGGAA			
aF5	CGTCATCACTGCAGACCTGT	A	455	This study
aR5	GGTAGCGCTCGACTACAGAC			
WF3	TGATGCTGTGGAACTCGGTC	A^w	346	This study
WR3	TCAACACACTGGCCACCTTT			
StarF5	AGCAATCCTTCTTTCGCGGA	A^*	143	This study
StarR5	TACTCTCCTTGGGCTAGCGT			

100% positive; the calculated copy number per reaction was approximately 34.

Multiplexed assay validation testing

All primer sets were multiplexed to make testing more efficient by combining individual tests into a single assay. Expected results when testing A, A^w, and A^* isolates are shown in Figure 1, where A, A^w, and A^* primers produced subpathotype-specific bands of 455, 346, and 143 bp, respectively. All subpathotypes produced a 561-bp band by the general Xac primer set. The multiplex assay was evaluated under hypothetical mixed-infection conditions, although mixed infections by different subpathotypes have not been reported to date. Each subpathotype was tested in the presence of the others in varying concentrations. It was determined that when all three subpathotypes are present at a low (200 fg/µl) concentration, all primer sets can produce distinct subpathotype-specific bands. In contrast, when a subpathotype is present at a low concentration, and the other two are present at a high (0.1 ng/µl) concentration, detection of the low-concentration subpathotype is affected negatively, and only a faint band is produced (data not shown). In general, the presence of all three subpathotypes does allow for detection of each; however, if there is a $\geq \sim 10$ C_t difference in concentration among the subpathotypes, detection of the low-concentration subpathotype will be impacted negatively.

Primers and reagents were sent to three outside laboratories for validation testing to evaluate assay analytical specificity, and a summary of results is shown in Table 4. Isolate information and results from individual laboratories are shown in Supplementary Tables S1, S2, and S3. In general, the multiplex cPCR assay was able to correctly determine subpathotype, save for a few exceptions. Cases were observed at both DPI and CIRAD, where isolates identified as A subpathotypes failed to produce the A-specific band; only the general Xac (561 bp) band was produced. CIRAD possesses an extensively curated collection of *X. citri* pv. *citri* isolates and observed this result among A strains belonging to Lineage 2, a less characterized lineage of citrus canker-causing strains. The identity of the "variant" A strain (3666; Supplementary Table S2) from DPI is unknown, and no follow-up testing was performed by DPI to identity this isolate. CIRAD also reported cross-reactions with a few non-*X. citri* pv. *citri* isolates. *X. citri* pv. *clitoriae* (LMG 9045), which is not a pathogen of citrus, produced the 561-bp Xac band only; *X. citri* pv. *clitoriae* was identified as an immediate ancestor of *X. citri* pv. *citri* (Patané et al. 2019), which most likely explains the presence of the general Xac marker. Additionally, two isolates (NCPPB 3213^T and NCPPB 1759) of *X. citri* pv. *bilvae* (previously *X. campestris* pv. *bilvae*), a pathogen of several rutaceous species in India, produced multiple bands, with NCPPB 1759 producing a banding pattern identical to A^w subpathotypes. These isolates were tested with the Xac ImmunoStrip and produced positive results (Supplementary Fig. S2), demonstrating cross-reactions with these non-*X. citri* pv. *citri* isolates, as A subpathotypes should produce positive results, whereas A^w and A^* subpathotypes should produce negative results. Given that *X. citri* pv. *bilvae* (NCPPB 1759) produced a banding pattern identical to A^w subpathotypes, the ImmunoStrip was able to identify this as a false positive exception, as true A^w subpathotypes would produce negative results. Based on validation ring test results, the calculated diagnostic specificity [# true negatives/(# true negatives + false positives) × 100%]/sensitivity [# true positives/(# true positives + false negatives) × 100%] for the A-, A^w-, and A^*-specific primer sets tested with DNA isolated from purified bacterial cultures was 100/79%, 98/100%, and 100/100%, respectively. Diagnostic sensitivity of the A-specific

TABLE 4

Summary of ring test results and calculated accuracies

Subpathotype	Total	Correct ID[a]	Percentage correct
A	38	30	79
A^*	22	22	100
A^w	12	12	100
Non Xac	54	53	98
Total	126	116	92

[a] Correct identification was defined as the production of two bands, one corresponding to the general Xac band and the other to the expected subpathotype-specific band. For the non-Xac strains, correct identification was failure to produce amplicons or a banding pattern identical to that of A, A^w, or A^*.

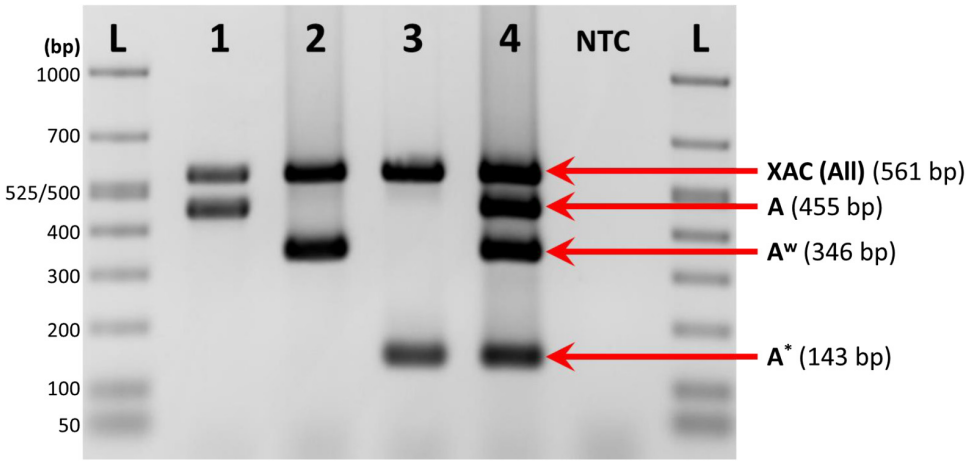

FIGURE 1

Gel image showing prospective results for the multiplexed *Xanthomonas citri* pv. *citri* pathotyping cPCR assay. Multiplex cPCR reactions were run on representative strains for each subpathotype, along with a mixture of all three subpathotypes and a no-template control (NTC). PCR products were resolved by electrophoresis using a 1.5% agarose gel prepared with 1× TAE buffer. A 100-base pair DNA ladder (BioVentures) was run alongside samples for amplicon-size comparison. 1) A (Xac A3213), 2) A^w (Xac 0053), 3) A^* (Xac 270), and 4) a mix of the three strains. Product sizes for the Xac (general), A, A^w, and A^* primers are 561, 455, 346, and 143 bp, respectively. As shown, isolates produce two bands, the 561-bp control band for Xac and a smaller second band unique to a specific subpathotype, allowing for differentiation.

primer set was reduced due to the false negatives produced for A Lineage 2 isolates. Diagnostic specificity for the A^w-specific primer set was reduced due to the false positive from *X. citri* pv. *bilvae* NCPPB 1759. When accounting for data from both ring testing and initial in-house evaluation, the calculated diagnostic specificity/sensitivity for the A-, A^w-, and A^*-specific primer sets was 100/86%, 98/100%, and 100/100%, respectively. Diagnostic sensitivity calculations included only isolates that had subpathotypes determined previously.

Plant sample testing

To test the applicability of the multiplex cPCR assay for routine diagnostic testing of citrus canker samples, 56 field samples received during fiscal years 2013 to 2018 and processed according to the bacterial streaming method were removed from −80°C storage at the PPCDL and tested by the multiplex pathotyping cPCR. Samples were selected to encompass a variety of hosts from areas of known outbreaks of A and A^w subpathotypes. A^* is not present in the United States currently; thus, no A^* samples were tested. The assay was able to provide subpathotype determinations for each sample, with few exceptions (96% positive). Some samples with high reported C_t values (>32) failed to produce bands indicative of subpathotype. Reported C_t values were based on the real-time PCR assay (Santillana et al. 2021), which was used as a screening tool to detect Asiatic citrus canker isolates. The lack of results observed with low-titer samples was inferred from the tenfold lower sensitivity of the cPCR assay. Low-titer samples with C_t values approaching the limit of detection for the real-time PCR assay would be missed by the less sensitive cPCR assay. However, known cross-reactions with the real-time PCR do produce high C_t values, which could represent false positives that would not be detected by the cPCR assay and artificially decrease the percent positive detection. High C_t values by real-time PCR are not indicative of a true citrus canker, as even small lesions produce C_t values < 32. Additionally, samples containing PCR inhibitors such as endogenous plant molecules or exogenous compounds from foliar sprays affected downstream cPCR results. This phenomenon was observed as decreases in C_t value when using diluted samples or the appearance or increased band intensity when DNA was extracted from leaf samples or cleaned using a commercial kit.

Sample age and quality affected downstream results for the real-time PCR and multiplex cPCR assays. This is mostly a consequence of sample collection and not the assays themselves. The most common issue observed by diagnosticians at the PPCDL was inhibition due to potential foliar sprays, as leaf tissue would appear to have spray residue. Samples would produce acceptable C_t values (<30) but no bands by cPCR. The bacterial streaming method is convenient, quick, and easy, but the crude nature of sample preparation does allow inhibitor carryover. Therefore, the bacterial streaming protocol was updated to help remove potential inhibitory substances. The new protocol was validated using diagnostic samples of A and A^w subpathotypes from different hosts and showed recovery of bands by reducing inhibition, as well as increasing band intensities in corresponding samples. A brief representation of results is shown in Figure 2. Two diagnostic samples comprising symptomatic leaf tissue that produced C_t values ~26 by real-time PCR, but no bands by cPCR upon initial testing, were retested after processing samples using both the original and new protocols for comparison. As shown in Figure 2, the new protocol was able to dramatically reduce inhibition to recover sample bands at relatively high intensities.

The new sample preparation protocol was also tested on poor-quality samples (overly necrotic, old, dried out, and/or soiled), and bands for subpathotype determination were produced (data not shown). Mock low-titer samples were also prepared (1:6 ratio of lesion to healthy tissue) and tested. Results revealed that bands could be produced from samples containing at least 1/6 the amount of infected tissue as stated in the protocol (data not shown), suggesting that low-titer true citrus canker samples should not present any issues when tested with the multiplex cPCR assay. Under real diagnostic conditions, even small pinpoint lesions were detectable by this method. The streaming protocol was also tested on fruit lesions. Unfortunately, inhibition from fruit samples could not be overcome; therefore, DNA extraction by a commercial kit is recommended. To test the utility of the entire process, a blind panel of five samples was tested by two diagnosticians at the PPCDL, using the new sample preparation upstream of the multiplex cPCR assay, to establish reproducibility in-house. Both diagnosticians produced accurate and congruent results (data not shown), suggesting high reproducibility with the assay.

DISCUSSION

Genetic and pathological differences among *X. citri* pv. *citri* strains yielded the delineation of subpathotypes and lineages, which were found to be distinguishable by pathogenicity assays (Vernière et al. 1998), genotyping techniques such as AFLP (Bui Thi Ngoc et al. 2010) or MLVA (Pruvost et al. 2014), or WGS (Gordon et al. 2015; Patané et al. 2019). In this study, a multiplex

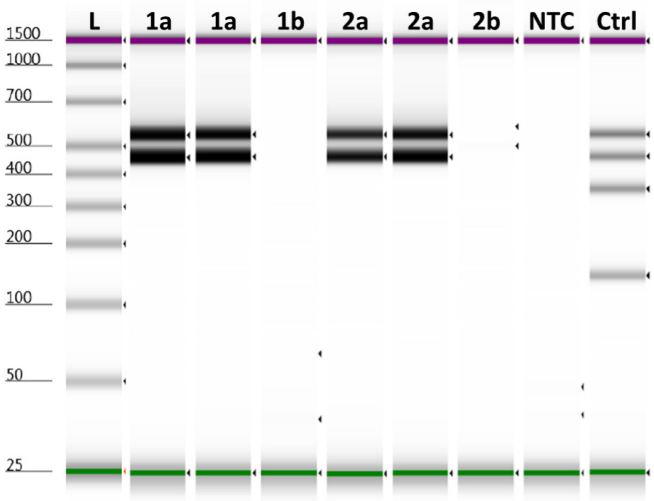

FIGURE 2
Representative cPCR results demonstrating improved sample preparation by reducing inhibition. Two different field samples (leaves showing symptoms of citrus canker) (1 and 2) submitted for diagnostic testing, which initially produced real-time PCR C_t values ~26 but no amplicons, were retested with the multiplex cPCR assay. Samples were processed by a, the updated bacterial streaming protocol utilizing Chelex 100 Chelating Resin and TE buffer in duplicate wells, or b, the original protocol in a single well. Samples were run with a no-template control (NTC) and a positive control (Ctrl) consisting of a mixture of DNA from all three subpathotypes. Amplicons were analyzed using the 4200 TapeStation System, D1000 ScreenTape, D1000 DNA Ladder (L) and D1000 Sample Buffer, according to the manufacturer's instructions. Inhibition experienced during routine diagnostic testing of these samples was real, and results were repeatable. Use of the new protocol recovered amplicons with significant intensity.

cPCR assay was developed to confirm the presence of Asiatic citrus canker pathogens and determine infecting subpathotypes. The assay correctly determined almost all A, A^w, and A^* isolates tested (high analytical specificity). It was found that isolates of subpathotype A Lineage 2, a poorly characterized lineage among the A strains (Gordon et al. 2015; Leduc et al. 2015; Patané et al. 2019; Pruvost et al. 2014, 2021), were detected with the control primer set (Xac F/R) but not the A-specific primer set (aF5/aR5). Attempts were made to design primers for this group by aligning sequences of Lineage 2 strains available in GenBank (NCPPB 3612, LG97, LG102, LE116-1, and LH37-1) (Richard et al. 2021) with A strain 306, A^*, and Texas isolates (A^w) (Munoz Bodnar et al. 2017) to find unique regions. Regions unique to Lineage 2 were not identified; however, regions shared and unique to all A lineages were found and used to design several primer sets. Feasibility testing with these primer sets showed cross-reactions with A^w or A^*. Other primer pairs were designed to target both Lineage 2 and A^w, or Lineage 2 and A^*; however, cross-reactions with nontargets were still observed.

Currently, A Lineage 2 strains have only been found in Southwest Asia (India and Bangladesh) and West Africa (Senegal, Mali, and Burkina Faso) (Gordon et al. 2015; Leduc et al. 2015; Patané et al. 2019; Pruvost et al. 2014, 2021). The agricultural and economic significance of Lineage 2 has not been widely examined (Pruvost et al. 2021), and Lineage 2 has not yet been reported in the United States; thus, identification of Lineage 2 strains is not a current priority for routine diagnostic testing at the PPCDL. Lineage 2 will produce canker symptoms and test positive for the general Xac marker (561-bp band). Negative results with the Xac ImmunoStrip assay may suggest the presence of a Lineage 2 strain, which could then be analyzed by other methods. Inability to identify Lineage 2 isolates with the A-specific primers does not detract from the usefulness of this assay. To improve this assay, inclusion of a test that can differentiate Lineage 2 isolates will be examined further in the future.

X. citri pv. *bilvae* is a poorly characterized pathogen of citrus (Bui Thi Ngoc et al. 2010) and related rutaceous genera that was originally reported to cause disease on Indian bael (*Aegle marmelos*, also known as Bengal quince, golden apple, Japanese bitter orange, stone apple, or wood apple) (Patel et al. 1953). *X. citri* pv. *bilvae* can be clearly distinguished from *X. citri* pv. *citri* by AFLP analyses and multi-locus sequence analysis (Bui Thi Ngoc et al. 2010). *X. citri* pv. *bilvae* did cross-react with this assay and produced a banding pattern indicative of an A^w subpathotype for one of the two isolates tested (NCPPB 1759); this isolate was pathogenic to Mexican lime and not alemow. According to information provided by the culture collection, NCPPB 1759 was isolated from *Limonia acidissima* (wood apple), whereas the type strain (NCPPB 3213^T) was isolated from *Aegle marmelos* (Indian bael). The worldwide distribution of *X. citri* pv. *bilvae* is unknown, and it may no longer exist in nature, as only these two isolates seem to appear in the literature, and no new isolates have been reported. If this is the case, one would not expect *X. citri* pv. *bilvae* to pose issues for this diagnostic.

Pathogenicity tests have shown that *X. citri* pv. *bilvae* can infect Mexican lime (isolates NCPPB 3213^T and NCPPB 1759) and alemow (only isolate NCPPB 3213^T) (Bui Thi Ngoc et al. 2010; Rybak et al. 2009; Sun et al. 2004), the hosts for A^w subpathotypes; however, symptomologies were clearly distinct when compared with A^w subpathotypes. *X. citri* pv. *bilvae* did not produce canker-like symptoms on Mexican lime, instead producing extensive water-soaked lesions, and showed differential pathogenicity on alemow, producing small water-soaked lesions with certain isolates (Bui Thi Ngoc et al. 2010). Observance of true canker symptoms is an effective initial screen against Mexican lime and alemow samples infected with *X. citri* pv. *bilvae*. As the current distribution (not reported in the United States currently) of this very low-impact pathovar in citrus has not been established, a banding pattern indicative of an A^w subpathotype for hosts other than Mexican lime and alemow would require further investigation, if not already prescreened based on symptoms. This would determine if it is a false positive from *X. citri* pv. *bilvae* or true infection from an A^w subpathotype. Use of the Xac ImmunoStrip can provide some insight, as *X. citri* pv. *bilvae* would test positive, and true A^w subpathotypes would test negative.

Overall, data suggest that this multiplexed cPCR assay is suitable for detection and subpathotype determination of *X. citri* pv. *citri* causing Asiatic citrus canker, as determined by the high analytical and diagnostic sensitivities and specificities for relevant isolates. This assay was evaluated using 146 unique *X. citri* pv. *citri* (A, A^w, and A^*), nine CBC-B, seven CBC-C, eight *X. euvesicatoria* pv. *citrumelonis* (causal agent of citrus bacterial spot), two *X. citri* pv. *bilvae*, 12 pathovars of *X. citri* that do not infect citrus, and 20 nonpathogenic *Xanthomonas* isolates. Confidence in this assay resulted in the PPCDL adopting this assay for subpathotype determination. The assay has been able to successfully determine subpathotype in the majority of samples received at the PPCDL, indicating high diagnostic sensitivity and specificity when testing infected leaf tissue samples; results were not obtained for samples lacking true citrus canker. The diagnostic sensitivity and specificity for the cPCR assay is 100% for diagnostic field samples presenting true citrus canker lesions. Additionally, no samples tested at the PPCDL have produced anomalous results associated with potential infection by an A Lineage 2, *X. citri* pv. *bilvae*, or *X. citri* pv. *clitoriae* strains, suggesting a very high diagnostic sensitivity and specificity. The assay was coupled downstream of a simplified extraction method that reduces time and costs associated with sample preparation. The updated protocol reduces inhibition associated with endogenous plant molecules and exogenous compounds, such as foliar sprays, and accommodates samples of varying age and quality; very old and/or poor-quality samples could still cause issues with this protocol. Leaf samples of decent quality that possessed true lesions (regardless of size, age, and amount) and were processed by the updated protocol have demonstrated 100% diagnostic sensitivity at the PPCDL. One limitation of the simplified protocol is that it does not work with fruit samples; thus, DNA extraction using a commercial kit is recommended. The multiplexed cPCR assay successfully produces amplicons from fruit samples processed by commercial extraction kits such as the DNeasy Plant Mini Kit (Qiagen). This assay has proven useful for detecting Asiatic citrus canker pathogens and determining the infecting subpathotype from symptomatic citrus tissue, noting its limitations for A Lineage 2 and *X. citri* pv. *bilvae* strains. This assay fulfilled the needs of stakeholders and CHRP for fast, user-friendly, and cost-effective diagnostics and has since been implemented at the PPCDL for the benefit of the U.S. citrus industry.

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