



# Genomics-Informed Molecular Detection of *Xanthomonas vasicola* pv. *vasculorum* Strains Causing Severe Bacterial Leaf Streak of Corn

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Accepted for publication 15 May 2019.

## ABSTRACT

*Xanthomonas vasicola* pv. *vasculorum* (syn. *X. campestris* pv. *vasculorum*) was initially identified as the causal agent of bacterial leaf streak of corn in South Africa. The pathovar *vasculorum* causes disease on sugarcane and corn, but a subset of these strains was noted for its increased disease severity in corn. This subset was reclassified as *X. campestris* pv. *zeae* in the early 1990s and was found to have slightly different biochemical and genetic properties than isolates from sugarcane. There has been an emergence of *X. campestris* pv. *zeae*-like strains of *X. vasicola* pv. *vasculorum* in both the United States and Argentina since 2010. We performed whole genome sequencing on U.S. isolates to confirm their identity. Informed by comparative genomics, we then developed specific TaqMan qPCR and loop-mediated isothermal amplification (LAMP) assays for the detection of this specific subset of *X. vasicola* pv. *vasculorum* strains. The qPCR 4909 assay was tested

against 27 xanthomonads (diverse representation), 32 DNA extractions from corn leaves confirmed as positive or negative for the bacterium, 41 *X. vasicola* pv. *vasculorum* isolates from corn in the United States and Argentina, and 31 additional bacteria associated with corn, sugarcane, or sorghum. In all cases the assay was shown to be specific for the *X. vasicola* pv. *vasculorum* isolates that cause more severe disease on corn. We then tested the LAMP 166 assay against the 27 xanthomonads and 32 corn leaf DNA samples, and we found this assay was also specific for this subset of *X. vasicola* pv. *vasculorum* isolates. We also developed a live/dead cells distinction protocol using propidium monoazide prior to DNA extraction for analyzing seed washes using these assays. These two detection assays can be useful for both diagnosticians and researchers to specifically identify the *X. vasicola* pv. *vasculorum* isolates that cause more severe symptoms on corn.

Bacterial leaf streak of corn, caused by *Xanthomonas vasicola* pv. *vasculorum*, emerged within the last decade on two new continents in addition to the country of South Africa, where it was described in the late 1940s. In the 1950s, South African officials observed that the disease caused little damage and did not affect plant development, and it was therefore not regarded as economically important (Coutingo and Wallis 1991). In the late 1980s in South Africa, however, bacterial leaf streak became a greater cause of concern as it frequently occurred both at breeding stations and in farming areas, leading to the withdrawal of a commercial maize variety due to susceptibility to the disease (Coutingo and Wallis 1991). Recently,

the disease was found in the United States, Argentina, and Brazil (Korus et al. 2017; Leite et al. 2019; Plazas et al. 2018; E. Sagata et al., *unpublished data*). The U.S. Department of Agriculture issued a statement in 2016 indicating that it did not consider the disease to be of quarantine significance for domestic or international trade (Boehland 2016).

A challenge to corn growers is identifying the disease for proper disease management. Symptoms of bacterial leaf streak occur on leaves of field (dent) corn, sweet corn, and popcorn, appearing as yellow-brown lesions with wavy margins following leaf veins (Coutingo and Wallis 1991; Lang et al. 2017). Late stage symptoms can be confused with a fungal disease of corn, gray leaf spot, caused by *Cercospora zeae-maydis*. Growers benefit from quick and accurate identification of disease-causing agents to make the most efficient management decisions, as fungicide applications have no effect on bacterial leaf streak. Additionally, not all *X. vasicola* pv. *vasculorum* strains cause severe disease in maize, making it important to identify those strains more likely to warrant a management decision.

Identifying *X. vasicola* pv. *vasculorum* can be challenging because it has been isolated from multiple hosts and has a complex taxonomic history. There are differences in disease severity among isolates on certain hosts, e.g., corn and sugarcane, but possibly not enough biochemical and physical differences to warrant different pathovar designations (Qhobela et al. 1990). A subset of isolates, originally classified as *X. campestris* pv. *vasculorum* and causing relatively severe symptoms on corn, were subsequently called *X. campestris* pv. *zeae* and could be differentiated from sugarcane isolates by restriction fragment length polymorphism, suggesting genomic differences between the strains (Coutingo and Wallis

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**Funding:** Funding was provided in part by the Farm Bill. This research was supported in part by the intramural research program of the U.S. Department of Agriculture, Animal and Plant Health Inspection Service. The findings and conclusions in this preliminary publication have not been formally disseminated by the U.S. Department of Agriculture and should not be construed to represent any agency determination or policy. D. J. Studholme acknowledges the support of COST Action CA16107 EuroXanth, supported by COST (European Cooperation in Science and Technology).

\*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary tables are published online.

The author(s) declare no conflict of interest.

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1991; Qhobela and Claflin 1992; Qhobela et al. 1990). Recent whole-genome analysis showed that these differences led to distinct phylogenetic clustering of corn isolates compared with isolates from sugarcane (G. Wu et al., *unpublished data*). Sugarcane isolates can still be found on corn, especially if the two crops are grown in the same geographical area, but it is of greater concern to growers if the strains causing severe symptoms on corn are present (Coutingo and Wallis 1991). The reclassification of *Xanthomonas* by Vauterin et al. (1995) subdivided *X. campestris* pv. *vasculorum* into two new taxa: *X. vasicola* pv. *vasculorum* and *X. axonopodis* *vasculorum*. Vauterin's description of new taxon *X. vasicola* pv. *vasculorum* was invalid, as it did not designate a pathotype strain. Nevertheless, most authors have subsequently followed Vauterin's nomenclature, and a recent review of the *X. vasicola* species taxonomy presents an updated formal description of the pathovar *X. vasicola* pv. *vasculorum* (Studholme et al. 2019). It should also be noted that Vauterin's taxonomic studies did not include *X. campestris* pv. *zeae* corn strains but did use a corn isolate of *X. vasicola* pv. *vasculorum* (LMG 8284 or NCPPB 206) that was not representative of the bacterium being described as *X. campestris* pv. *zeae* (Vauterin et al. 1992); thus the taxonomic position of *X. campestris* pv. *zeae* corn strains was not addressed during the Vauterin et al. (1995) reclassification.

Here, we sequenced the whole genome of strain SAM119. This strain was historically classified as *X. campestris* pv. *zeae* and more recently was proposed as the type strain of *X. vasicola* pv. *vasculorum* (Lang et al. 2017; Studholme et al. 2019). We compared it to genomes of nine *X. vasicola* isolates from multiple hosts, with the goal to design real-time PCR and loop-mediated isothermal amplification (LAMP) assays for fast and accurate detection of the strains known to cause more severe disease on corn. We designed two assays, a field deployable assay (the LAMP assay) for bacterial streaming samples taken from leaf lesions, and a more sensitive real-time PCR assay to detect *X. vasicola* pv. *vasculorum* in total DNA extracts from leaf material or seed wash. A third goal was to develop an assay that could be used to test seeds directly and distinguish live versus dead cells, which led to a propidium monoazide (PMA) protocol prior to seed wash DNA extraction and detection.

## MATERIALS AND METHODS

**Bacterial strains and samples.** Twenty-seven strains representing a variety of *Xanthomonas* spp. were obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB) to evaluate the specificity of the real-time PCR and LAMP assays. Strain identity was confirmed by amplifying and sequencing at one or more of the following genes: *gyrB*, *atpD*, and *dnaK* (Ah-You et al. 2009; Parkinson et al. 2007). Specificity testing also included *Dickeya solani* and *Ralstonia solanacearum* strains plus 32 DNA extracts from corn leaf samples with bacterial leaf streak-like symptoms. Presence or absence of *X. vasicola* pv. *vasculorum* in these samples was determined by PCR, Sanger sequencing, and phylogenetic analysis as part of confirmatory diagnostics by the APHIS-PPQ-S&T-CPHST-Beltsville laboratory. The real-time PCR assay was tested against a second collection of bacterial organisms. This collection consisted of 72 bacterial organisms isolated from corn, sorghum, or sugarcane, with 41 *X. vasicola* pv. *vasculorum* isolates from corn representing multiple U.S. states and Argentina.

**Genome sequencing, assembly, and comparison.** Four *Xanthomonas* genomes were sequenced, three maize isolates originating from U.S. states (Xvv 645, Xvv 681, and Xvv 715) and the strain SAM119 isolated from maize in South Africa (all *X. campestris* pv. *zeae*-like strains). The U.S. maize isolates were collected in 2016 from Kansas (Xvv 645), Colorado (Xvv 681), and Minnesota (Xvv 715).

DNA was extracted from overnight cell cultures grown in nutrient agar broth using a CTAB method for strains Xvv 645, Xvv 681, Xvv

715, and a Blood & Cell Culture DNA Maxi Kit (Qiagen) for strain SAM119. DNA was fragmented to roughly 550 bp sizes using a M220 sonicator (Covaris, Woburn, MA) and prepared using the TruSeq Nano DNA Library Prep kit (Illumina, San Diego, CA) for 300-bp paired end sequencing on a MiSeq platform (Illumina). For PacBio sequencing, DNA from SAM119 was submitted to the University of Maryland School of Medicine, Institute for Genome Sciences, where it underwent long-insert PacBio library preparation before PacBio RS II sequencing.

Genomes were assembled using SPAdes 3.10.1 (Bankevich et al. 2012) and Pilon v1.22 (Walker et al. 2014). Draft genomes were annotated with GeneMarkS+ using the National Center for Biotechnology Information (NCBI) Prokaryotic Genome Annotation Pipeline. Raw sequencing files were deposited to the Sequence Read Archive and assembled genomes deposited to GenBank: Xvv 645 (SRR8239205, PYJJ00000000), Xvv 681 (SRR8240252, PYJK00000000), Xvv 715 (SRR8240255, PYJL00000000), and SAM119 (name: Xv 416, SRA PacBio SRR8240386 and Illumina SRR8240385). Genomes were obtained from GenBank via the NCBI web portal for phylogenetic analysis using REALPHY (Bertels et al. 2014).

The genome of the *X. vasicola* pv. *vasculorum* pathotype strain SAM119 (CP028127) (Lang et al. 2017) was compared with *X. vasicola* pv. *vasculorum* strain NCPPB 206 (AKBM01000000), *X. vasicola* pv. *vasculorum* strain NCPPB 1326 (AKBK01000000), *X. vasicola* strain NCPPB 1396 (JSBX01000000), *X. vasicola* strain NCPPB 1241 (JSBV01000000), *X. campestris* pv. *musacearum* strain NCPPB 2005 (AKBE01000000), *X. campestris* pv. *arecae* strain NCPPB 2649 (JSBP01000000), and *X. oryzae* pv. *oryzae* strain MAFF 311018 (NC\_007705) using the Seq\_diff program (Stulberg et al. 2015) to identify sequences unique to SAM119. Unique regions were screened in local BLAST (Altschul et al. 1990) searches using Geneious software v10 (Kearse et al. 2012) and compared with other *X. vasicola* genomes for inclusivity and exclusivity. For visualization, alignments of raw Illumina reads to SAM119 were performed using BWA-mem (Li and Durbin 2009) and SAMtools (Li et al. 2009) prior to using the Integrative Genomic Viewer v2.4.19 (Thorvaldsdóttir et al. 2013). Primers and probe for the TaqMan assay were designed using Geneious software v10 and the Integrated DNA Technologies (IDT, Coralville, IA) website, with design parameters targeting a GC = 45 to 60%, T<sub>m</sub> = 60°C, and primer length = 18 to 26 bp. Primers for the LAMP assay were designed using PrimerExplorerV4.

**Real-time PCR.** Two labs participated in the validation of the TaqMan real-time PCR assay: the method development lab and the testing lab. In the method development lab, real-time PCR assays were performed in a 25 µl volume with 1× PCR buffer (Invitrogen, Waltham, MA), Platinum Taq polymerase (Invitrogen) 1.25 U per reaction, 0.24 µM each of primers 4909\_F and 4909\_R, 0.12 µM of probe 4909\_probe, 3 mM MgCl<sub>2</sub> (Invitrogen), 0.3 mM of dNTPs (Sigma Aldrich, St. Louis, MO), 50 nM ROX Reference Dye (Invitrogen), and 2 µl of template DNA. Reactions were performed in a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA). Cycling conditions were 95°C for 2 min, 40 cycles of 95°C for 30 s and 60°C for 30 s, with the threshold set to automatic. The second testing laboratory performed real-time PCR assays in a QuantStudio 3 Real-Time PCR system (Applied Biosystems) using the PrimeTime Gene Expression Master Mix (IDT) with ROX as a passive reference dye. Cycling parameters and primer amounts were the same as in the method development lab.

**LAMP.** LAMP reactions were carried out with a total volume of 25 µl containing 0.2 µM each of primers 166\_F3 and 166\_B3, 1.6 µM each of primers 166\_FIP and 166\_BIP and 0.8 µM of primer 166\_LoopF, 15 µl of Isothermal Master Mix Iso-001 (Optigene, Horsham, UK), 3 µl of molecular grade water, and 2 µl of template DNA. The LAMP assay was run at 65°C for 30 min with a preheat step at 65°C for 1 min (Genie III, Optigene). Threshold was set at

0.02 fluorescence ratio for the amplification rate (derivative of amplification curve).

**Determination of limit of detection, linearity, and amplification efficiency.** Isolate Xvv 681 and pathotype strain SAM119 (syn. Xcz 416) were grown in nutrient broth at 30°C with shaking for 24 h. Tenfold and half-log dilutions spanning a five-log range were prepared using sterile water and incubated at 95°C for 10 min in a thermomixer (Eppendorf, Hamburg, Germany). Two microliters from each dilution were used as a template in the real-time PCR assays and run in triplicate. Prior to heat treatment, aliquots from the  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$ , and  $10^{-7}$  dilutions were spread-plated on nutrient agar plates and incubated at 30°C overnight to determine colony forming units (CFU) per ml. Standard curves were constructed relating CFU to Ct values, and limit of detection, linearity, and amplification efficiency were determined. Three different strains of *X. vasicola* pv. *vasculorum* were used and each dilution was run in triplicate.

To evaluate any plant matrix effect, tenfold serial dilutions of the heat-treated cultures were prepared in DNA extracted from healthy corn leaves (DNeasy Plant Mini Kit, Qiagen), and real-time PCRs were set up as previously described, in duplicate. Limits of detection, linearity, and amplification were determined and compared with those dilutions in water.

**PMA seed wash assay.** Corn seeds were soaked for 8 h in phosphate buffered saline (PBS)-buffer with  $10^8$  CFU/ml *X. vasicola* pv. *vasculorum* in a 10°C cold room, dried on screens in a stack dryer to 11% moisture with room temperature air (22 to 24°C), and stored at room temperature for 9 months prior to use. After storage, 15 seeds were washed in 10 ml of PBS for 4 h with gentle agitation, after which three 1-ml aliquots were removed from each seed wash and each treated with a different DNA extraction protocols. In the first protocol, cells were pelleted by centrifugation for 2 min at  $14,000 \times g$ , suspended in 400  $\mu$ l of PBS plus 100  $\mu$ l of 5 $\times$  PMA Enhancer for Gram negative bacteria, and treated with 50  $\mu$ M PMA following the manufacturer's protocol (Biotium, Hayward, CA) including room temperature incubation in the dark for 10 min with agitation (700 rpm in thermomixer, Eppendorf), 15 min in a light box (PMA-Lite LED Photolysis device, Biotium), centrifugation for 5 min at  $5,000 \times g$  to pellet cells and remove supernatant and DNA extraction by the standard protocol of a Soil Extraction kit using buffer 1 (Macherey-Nagel, Düren, Germany). In the second protocol, DNA was extracted by pelleting cells for 2 min at  $14,000 \times g$  and suspending in lysis buffer with no prior PMA treatment (no PMA treatment). In the third protocol, serial dilutions were prepared and plated on nutrient agar to determine CFU/ml. PMA and non-PMA-treated seed washes were used as template for real-time PCR and LAMP assays as described previously. Experiments were repeated three times, and qPCRs run in triplicate. For negative controls, strains Xvv 715 and Xvv 681 were grown overnight in nutrient broth at 30°C. One milliliter of overnight culture was transferred to a 1.5-ml tube and incubated for 10 min at 95°C, shaking at 700 rpm in a thermomixer (Eppendorf). Cells proceeded through the DNA extraction protocols as described for the seed washes. Each DNA extraction was tested by qPCR in duplicate.

## RESULTS

The genomes of U.S. corn isolates clustered within a subgroup of the *X. vasicola* pv. *vasculorum* clade based on REALPHY analysis (Fig. 1). A genome comparison of the historical isolate SAM119 to the *X. vasicola* pv. *vasculorum* sugarcane reference strain NCPBB 1326 revealed a unique 721-bp region in the SAM119 genome. BLAST analyses confirmed the 721 bp region was also present in isolates Xvv 645, Xvv 681, Xvv 715, and Xvv NE744. No corresponding match for this region was found in the *X. vasicola* pv. *holcicola*, *X. campestris* pv. *arecae*, *X. vasicola*, or *X. campestris* pv. *musacearum* genomes available in GenBank at the time (Fig. 2). The 721-bp region contains the terminal end of a predicted

filamentous hemagglutinin N-terminal domain containing protein, and primers and probe were designed to the coding region for both TaqMan qPCR (qPCR 4909) and LAMP (LAMP 166) assays (Table 1). A complete genome of SAM119 and three draft genomes of Xvv 645, Xvv 681, and Xvv 715 were deposited to NCBI as a result of this study.

The qPCR 4909 and LAMP 166 assays were validated against positive and negative environmental (leaf) DNA samples and a culture collection. The qPCR 4909 assay correctly identified the positive (18/18) and negative (0/14) leaf samples from nine different states. The LAMP 166 assay identified all but one low titer leaf sample (17/18) and none of the negative samples (0/14). The culture collection consisted of 31 out-group strains. The two assays were evaluated against 27 different *Xanthomonas* species or pathovars, *D. solani*, and a *R. solanacearum* strain. No off-target organisms were detected (Supplementary Table S1).

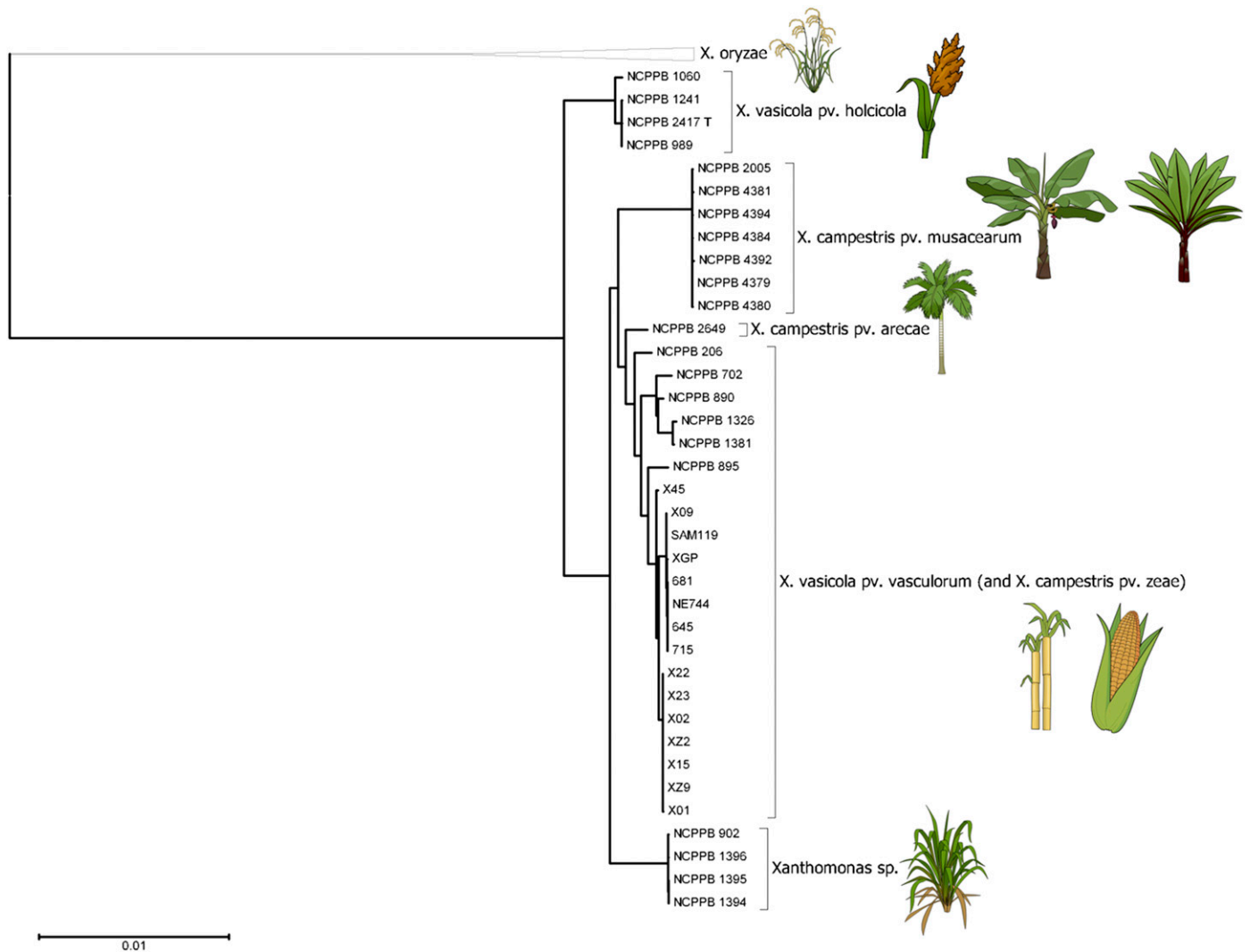
The assays were tested for limit of detection and efficiency. Additionally, the qPCR 4909 assay was tested for matrix effects in water and corn backgrounds (DNA from corn leaf). The efficiency of the assay was between 90 to 93% in water and near 100% in the corn background (Fig. 3). The limit of detection was approximately  $1 \times 10^3$  CFU/ml in water and  $2 \times 10^3$  CFU/ml in the corn DNA background. The semiquantitative LAMP 166 assay had a limit of detection near  $1 \times 10^4$  CFU/ml in a water background to mimic bacterial streaming conditions (Table 2). Detection time ranged from 5 min for the high concentrations to 11 to 15 min for the low concentrations.

A live/dead cells distinction assay was developed to accompany the molecular diagnostics of seeds. PMA was added to seed wash exudate prior to DNA extraction. Cycle threshold (Ct) values using the qPCR 4909 assay for non-PMA-treated samples were 25.506 ( $\pm 0.071$ ), 23.195 ( $\pm 0.865$ ), and 26.324 ( $\pm 0.073$ ), whereas with PMA treatment those values were 34.632 ( $\pm 0.515$ ), 32.491 ( $\pm 0.141$ ), and 38.805 ( $\pm 0.752$ , two out of three technical replicates). Plating of the same seed wash estimated live bacterial populations of  $3 \times 10^3$ ,  $3.2 \times 10^4$ , and  $1.01 \times 10^3$  CFU/ml remaining after treatment, respectively. Using the LAMP 166 assay, detection times without PMA were at 05:45, 05:15, and 05:45 min:s, whereas with PMA the values were slightly delayed at 08:30, 08:30, and 12:30, respectively. Additionally, cells were grown overnight, heat treated, and then treated either with or without PMA prior to DNA extraction. Ct values using the qPCR 4909 assay of non-PMA-treated cells were 20.569 ( $\pm 0.050$ ) and 20.531 ( $\pm 0.001$ ), whereas with PMA treatment the values were 34.742 ( $\pm 0.014$ ) and 39.434 (one replicate only).

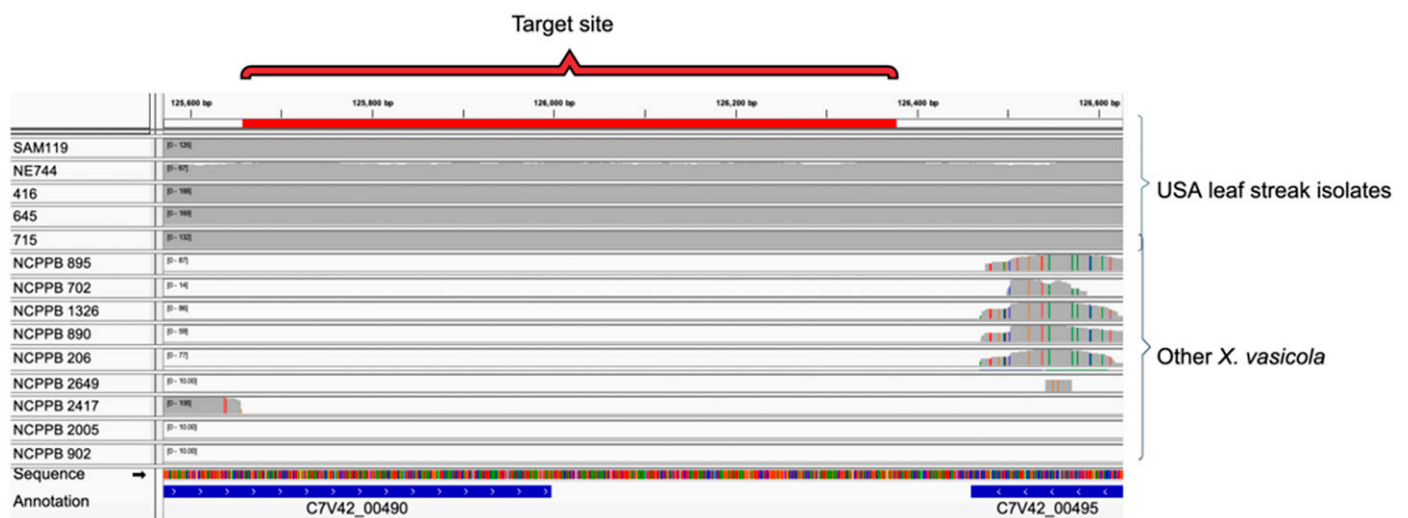
A second laboratory tested the qPCR 4909 assay using isolates from their collection. The qPCR buffer was different, but they correctly detected 41 of 41 *X. vasicola* pv. *vasculorum* strains from corn from multiple U.S. states and Argentina. Thirty-one additional out-group bacterial pathogens were tested, with one *Pantoea* sp. and two unknowns giving Ct values between 30 and 35. Twelve out-group isolates produced Ct values between 35 and 40, but the target strains were well below a Ct of 30 (Supplementary Table S2).

## DISCUSSION

The causal agent of corn bacterial leaf streak, *Xanthomonas vasicola* pv. *vasculorum*, has recently emerged in the United States, Argentina, and Brazil (Korus et al. 2017; Leite et al. 2019; Plazas et al. 2018; E. Sagata et al., unpublished data). In this study, we completed the genome of the South African pathotype strain, SAM119 (Lang et al. 2017) and contributed three draft genomes of U.S. isolates to public databases. Additionally, with whole-genome single nucleotide polymorphism phylogenetic analysis, we were able to further delineate the *X. vasicola* clade into groups that correspond with pathogenicity data. Interestingly, the corn isolates found in the United States and SAM119 formed a subgroup within the *X. vasicola* pv. *vasculorum* clade, consistent with other findings



**Fig. 1.** Single nucleotide polymorphism-based phylogenetic analysis of *Xanthomonas vasicola* and closely related *Xanthomonas* strains using the program REALPHY. The U.S. isolates (681, NE744, 645, and 715) cluster with SAM119, historically *X. campestris* pv. *zeae*, within the *X. vasicola* pv. *vasculorum* clade. A clade from X45 to X01 contains all *X. campestris* pv. *zeae*-like strains but not all strains isolated corn, as NCPPB 206 does not cluster. Similarly, NCPPB 895, isolated from sugarcane, groups closer to corn strains than sugarcane. The other *X. vasicola* strains clustered with strains isolated from similar hosts.



**Fig. 2.** The 721-bp target genomic sequence is absent from other *Xanthomonas vasicola* genomes. The image shows part of an alignment of Illumina genomic shotgun sequencing reads aligned against the reference sequence of the SAM119 chromosome (GenBank CP028127.1). Each bacterial strain is represented by a panel containing a plot of coverage depth along the genome. The target region corresponds to positions 125,656 to 126,376 and is highlighted in red. The range of depths (i.e., the scale of the y-axis) is indicated for each bacterial strain toward the left of the plots in square brackets. The positions of two protein-coding genes are indicated along the bottom of the plot. These are locus tag C7V42\_00490, predicted to encode a large protein containing hemagglutination activity domains, and locus tag C7V42\_00495, predicted to encode type I toxin-antitoxin system SymE family toxin.

(G. Wu et al., unpublished data). This grouping informed the comparative genomics to mine the genomes for specific sequences to identify these particular strains (formerly *X. campestris* pv. *zeae*). We focused on a specific sequence that includes the 3' end of a predicted filamentous hemagglutinin (FHA) N-terminal domain-containing protein for detection assay design (Fig. 2). FHA proteins can mediate bacterial adherence and colonization, suggesting a potential functional role for this sequence and one that could remain conserved (Relman et al. 1989). Rapid TaqMan qPCR and LAMP assays were designed to this sequence for the specific detection of these strains.

A PCR protocol consisting of two independent reactions was previously developed for the detection of *X. vasicola* pv. *vasculorum* (Lang et al. 2017). The two assays, in combination, specifically detected all strains in the *X. vasicola* pv. *vasculorum* clade, including those strains which infect sugarcane. Our goal was to specifically detect only the strains known historically as *X. campestris* pv. *zeae*, and furthermore do so on a qPCR platform. Attempts to convert the thirty-cycle PCR reactions (Lang et al. 2017) to forty-cycle qPCR reactions for increased sensitivity resulted in off-target effects, which led us to perform comparative genomics to identify a new target region for assay development. In silico analysis indicates that the 721-bp unique region is conserved in the newly released *X. vasicola* pv. *zeae* genomes from South Africa (Sanko et al. 2018), and the two assays should therefore detect all strains between X45 and X01 in the phylogenetic tree (Fig. 1).

The objective of this study was to develop tools for detection and diagnostics, not to address taxonomy, which is discussed elsewhere (Lang et al. 2017; Studholme et al. 2019). Nevertheless, our results do confirm the presence of a monophyletic clade encompassing the recent U.S. corn isolates along with historical “*X. campestris* pv. *zeae*” strains. Furthermore, our results confirm that this corn-associated clade falls within the radiation of strains classified as

*X. vasicola* pv. *vasculorum* isolated from sugarcane and (in the case of NCPPB 206) corn. In other words, these strains causing severe disease on corn should be considered a sublineage within the diversity of *X. vasicola* pv. *vasculorum*. There is no phylogenetic basis for splitting them into a separate taxon (*X. vasicola* pv. *zeae*); furthermore, their overlapping host-ranges further undermines any case for taxonomic splitting *X. campestris* pv. *zeae* away from *X. vasicola* pv. *vasculorum*. The molecular assays designed in this study, however, can distinguish this sublineage from other *X. vasicola* pv. *vasculorum* strains.

The qPCR 4909 and LAMP 166 assays successfully discriminated positive from negative samples. The qPCR 4909 assay was tested against a bacterial culture collection in the method development lab and another culture collection in a second testing laboratory. The assay remained specific in determining positive and negative screenings in two different labs. The higher rate of Ct values in the mid to upper 30s by the second testing lab could be due to the different master mix and machine used. Attempts to use the same master mix were unsuccessful across platforms. Because the qPCR 4909 assay

TABLE 2. Loop-mediated isothermal amplification assay run on serial dilutions of *Xanthomonas vasicola* pv. *vasculorum* cells from SAM119 and Xvv 681<sup>a</sup>

CFU/ml	<i>X. vasicola</i> pv. <i>vasculorum</i> % detected	Average time (min:s)
10 <sup>7</sup> –10 <sup>8</sup>	100 (4/4)	5:15
10 <sup>6</sup> –10 <sup>7</sup>	100 (14/14)	5:43
10 <sup>5</sup> –10 <sup>6</sup>	100 (26/26)	7:29
10 <sup>4</sup> –10 <sup>5</sup>	96 (25/26)	11:12
10 <sup>3</sup> –10 <sup>4</sup>	29 (7/24)	15:38
0	0 (0/14)	n/a

<sup>a</sup> Total detected samples for each log range of cell concentrations were reported as percent detected.

TABLE 1. Primers and probe sequence and target for TaqMan PCR and loop-mediated isothermal amplification (LAMP)

Primer	Sequence 5'-3'	Amplicon size (bp)	Target
Real-time PCR		139	Filamentous hemagglutinin N-terminal domain-containing protein
4909_F	CTTCTATCTTGGATCCGTCGG		
4909_R	GCGGGAATATTAATCTGGCG		
4909_probe	/56-FAM/ATT AAG CGC /ZEN/GGC ATC TGT CAA CC/3IABkFQ/		
LAMP		183	Filamentous hemagglutinin N-terminal domain-containing protein
166_F3	TTGGATCCGTCGGAAGTC		
166_B3	CGGAAGTTCAGTAAGCAAGA		
166_FIP	GCCGCGCTTAATGATTCAATTGAATTCCTTGACAACATCTGGATT		
166_BIP	CCTGTGCCGCCAGATTTTAACAGGCTTACCACTCAGTTG		
166_loopF	GAAAATTCTGGCGGAAAGTTAAGC		

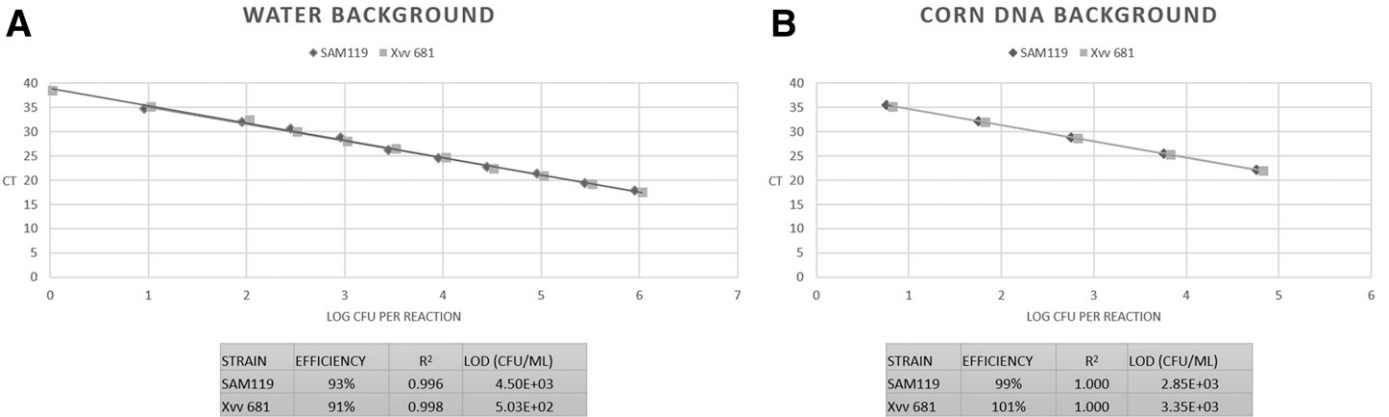


Fig. 3. TaqMan qPCR assay run on serial dilutions of *Xanthomonas vasicola* pv. *vasculorum* cells from SAM119 and Xvv 681. **A**, Serial dilution with a water background with calculated efficiency, R<sup>2</sup> value, and limit of detection (LOD). **B**, Serial dilution with corn leaf total DNA extract as a background with calculated efficiency, R<sup>2</sup> value, and LOD. All data points are an average of three technical replicates.

was run on pure cultures, it was easy to define a lower Ct threshold for true positive samples in the second testing laboratory. We recommend using a Ct cutoff of 30 when testing pure cultures. The qPCR 4909 assay also successfully detected samples extracted from corn leaf material with varying titers. Based on the detection limit experiments and the results from the environmental samples, we recommend establishing a Ct cutoff of 37 when testing total extracted DNA with a corn background. The LAMP 166 assay was only tested in the method development lab. A threshold of 0.02 was used to reduce false-positive values that sometimes occur early in a LAMP reaction.

The assays developed here can be used by diagnostic laboratories and researchers alike to quickly identify the *X. vasicola* pv. *vasculorum* strains that have historically produced more severe symptoms on corn. Moreover, incorporating PMA into the DNA extraction protocol from seed wash provided an effective method to quickly determine live versus dead cells using qPCR. This protocol may be useful to measure viable levels of *X. vasicola* pv. *vasculorum* on seeds to determine whether it meets a biologically relevant, predetermined threshold. The LAMP assay could be used following bacterial streaming and is portable, allowing potential use outside of the laboratory.

## ACKNOWLEDGMENTS

We thank Jillian Lang and Jan Leach for scientific discussions and providing strains for testing, and everyone involved in the 2016 corn field survey for bacterial leaf streak.

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