Sensitive, Secure Detection of Race 3 Biovar 2 and Native U.S. Strains of *Ralstonia solanacearum*

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Abstract

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Detecting and correctly identifying *Ralstonia solanacearum* in infected plants is important because the race 3 biovar 2 (R3bv2) subgroup is a high-concern quarantine pathogen, while the related sequevar 7 group is endemic to the southeastern United States. Preventing accidental import of R3bv2 in geranium cuttings demands sensitive detection methods that are suitable for large-volume use both onshore and offshore. However, detection is complicated by frequent asymptomatic latent infections, uneven pathogen distribution within infected plants, pathogen viable-but-not-culturable state, and biosecurity laws that restrict transport of R3bv2 strains for diagnosis. There are many methods to detect R3bv2 strains but their relative utility is unknown, particularly in the realistic context of infected plant hosts. Therefore, we compared the sensitivity, cost, and technical complexity of several assays to detect

and distinguish R3bv2 and sequevar 7 strains of *R. solanacearum* in geranium, tomato, and potato tissue in the laboratory and in naturally infected tomato plants from the field. The sensitivity of polymerase chain reaction (PCR)-based methods in infected geranium tissues was significantly improved by use of Kapa3G Plant, a polymerase with enhanced performance in the presence of plant inhibitors. R3bv2 cells were killed within 60 min of application to Whatman FTA(R) nucleic acid-binding cards, suggesting that samples on FTA cards can be safely transported for diagnosis. Overall, culture enrichment followed by dilution plating was the most sensitive detection method (10^1 CFU/ml) but it was also most laborious. Conducting PCR from FTA cards was faster, easier, and sensitive enough to detect approximately 10^4 CFU/ml, levels similar to those found in latently infected geranium plants.

Ralstonia solanacearum is the causal agent of bacterial wilt, also known as Southern wilt of geranium, brown rot of potato, and Moko disease of banana (7). The bacterium is soilborne and has an exceedingly wide host range that includes species from over 50 plant families (7,16). Several systems have been used to classify *R. solanacearum*, including races based loosely on host range; biovars based on biochemical properties; and, most recently, a phylotype-sequevar system based on phylogenetics, with four phylotypes that correspond to strain geographic origins and dozens of sequevars based on the *egl* gene sequence (7). Phylotype II, sequevar 1 strains of *R. solanacearum* are historically and for regulatory purposes known as race 3 biovar 2 (R3bv2). This subgroup attacks economically important hosts such as potato, tomato, and geranium and is adapted to cooler temperatures than most *R. solanacearum* strains (7). R3bv2 is listed as a quarantine pest in Europe and Canada and is a U.S. Select Agent pathogen. Select Agent pathogens are considered to pose particularly high risks to humans or agriculture and they are subject to very strict regulations (55). *R. solanacearum* R3bv2 has been repeatedly introduced to the United States and Canada, raising concerns that the pathogen will become established and affect agricultural production in northern countries.

To address this concern, much effort has been devoted to developing quick and reliable methods to detect *R. solanacearum*

(10,11,12,15,20,23,36,41,42,50). These assays include culture-based methods using semiselective media or enrichment culture; serological methods such as lateral-flow devices (ImmunoStrip) and enzyme-linked immunosorbent assay (ELISA); nucleic acid-based methods including direct polymerase chain reaction (PCR), real-time PCR, and loop-mediated isothermal amplification (LAMP); and various combinations of these. No single method offers optimal sensitivity, accessibility, and speed, particularly in the context of infected plants in remote production facilities.

Culture-based methods rely on the isolation and growth of the target pathogen (10). These methods can be highly sensitive and they have the important advantage of yielding a living culture that can be used for additional confirmatory analyses such as pathogenicity tests and for archiving. However, their usefulness is limited by the fact that *R. solanacearum* is relatively slow growing, has an undistinctive colony morphology, is significantly out-numbered by saprophytic microbes present in field samples (39), and can become viable but not culturable (56). Further, culturing bacteria from plant materials may not yield pathogen colonies because *R. solanacearum* populations are not evenly distributed in plants (52). Finally, the bacterium can undergo phenotypic conversion in culture, which results in an atypical colony morphology that is hard to recognize (7).

Serological detection methods are fast and easy but they cannot discriminate between living and dead pathogen cells, which is critical for quarantine decisions (7). Furthermore, existing serological methods lack specificity because commercially available tests use antibodies that cannot distinguish R3bv2 from other *R. solanacearum* strains. This is important because non-R3bv2 strains of *R. solanacearum* are endemic and widespread in the United States and, like R3bv2 strains, they also infect tomato, potato, and ornamentals. In addition, existing immunological assays are prone to weak false positives, which can trigger costly and unnecessary quarantine responses (7,10,14).

Nucleic acid-based detection methods theoretically offer sensitivity, specificity, and speed; therefore, many diagnostic tools for *R. solanacearum* involve various forms of PCR (10,12,27,35,37,38,48,50,51,54,58). Targets for amplification include ribosomal

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genes, the internal transcribed spacer region, fragments of unknown function, and virulence-associated genes such as *egl*, *hrpB*, and *fliC* (11,25,40,46). Like serological methods, PCR-based assays cannot discriminate between living and dead pathogens. Further, although nucleic acid-based detection methods work very well on pure DNA or bacterial cells in water, the DNA polymerases used in PCR are susceptible to inhibition by natural compounds in soil or plant tissue. Therefore, laborious and costly DNA extraction and purification is required to achieve high levels of sensitivity (12,29,31). This is a major disadvantage when large numbers of plants must be screened quickly.

Because R3bv2 strains have been accidentally introduced to North America and Europe in infected geranium cuttings (21,24,49,59), geranium producers and regulatory entities need effective protocols to rapidly identify infected plants at production sites in the highland tropics. The DNA-based assays that can quickly distinguish cool-tolerant R3bv2 strains from tropical *R. solanacearum* strains require equipment and technical expertise that are generally not available onsite. Quarantine and Select Agent regulations restrict and slow the transport of suspect samples. Therefore, we need a way to safely transport DNA from potentially infected plants to diagnostic labs.

Little is known about the relative sensitivity, reliability, and technical complexity of the many existing assays for the detection of R3bv2. A recent analysis compared several assays to detect *R. solanacearum* cells in ground potato tuber tissue, including culture, ImmunoStrip, ELISA, and PCR assays with various primers (30). However, this analysis did not determine assay sensitivity in tomato or geranium tissue, which contain more potent PCR inhibitors than potato tubers (12).

Bacterial wilt is also a serious problem for tomato growers around the globe. In the United States, the disease is usually caused by *R. solanacearum* phylotype II, sequevar 7 (9), which is endemic to the southeastern United States. Current Animal and Plant Health Inspection Service (APHIS) regulations consider all strains of the pathogen as Select Agent unless they are proven not to be R3bv2, which poses challenges for diagnosticians, plant breeders, and field plant pathologists working to manage bacterial wilt (55). Fast and reliable protocols are needed to reliably distinguish *R. solanacearum* R3bv2 from sequevar 7 strains in wilting, field-grown tomato plants.

To address these important practical challenges, we optimized a protocol to detect R3bv2 in tomato and geranium tissue, distinguish R3bv2 from endemic *R. solanacearum* strains, and allow secure transport of samples from the field to labs for diagnosis. We immobilized bacteria on Whatman FTA(R) nucleic acid-binding cards to kill the cells and bind their DNA for secure transport and then used a specialized DNA polymerase that is tolerant of plant inhibitors. This protocol was validated with R3bv2 and endemic sequevar 7 strains in challenging samples such as geranium stem extracts, artificially infected plants, and naturally infected field samples. In parallel, the same samples were analyzed using dilution plating, direct PCR, and enrichment in semiselective medium followed by either PCR or serological detection using ImmunoStrips. The various methods were compared with respect to sensitivity, speed, cost, and ease of use.

Material and Methods

Bacterial strains and culture. This study used R3bv2 strain UW551, which is typical of the essentially clonal population of globally distributed brown rot strains (4,5), and sequevar 7 strain K60, which is closely related to currently endemic strains in the southeastern United States (44,61). To facilitate bacterial enumeration, experiments were conducted with *R. solanacearum* strains K60-rif (phylotype II, sequevar 7) and UW551-rif (R3bv2; phylotype II, sequevar 1), which are resistant to rifampicin (52) and have wild-type virulence (34) (Table 1). Strains were maintained on 1.8% agar casamino acid peptone glucose (CPG) plates (17) supplemented with 2,3,5-triphenyl tetrazolium chloride (TZC) at 20 mg/liter and rifampicin at 25 mg/liter. Liquid cultures of *R. solanacearum* were grown in CPG broth (rifampicin at 25 mg/liter) overnight at 28°C, with shaking at 250 rpm.

Serial dilutions of *R. solanacearum* strains K60-rif and UW551-rif were prepared as follows: strains were grown on CPG broth supplemented with rifampicin (25 mg/liter) overnight and cells were collected by centrifugation and resuspended in sterile water. Bacterial suspensions were adjusted spectrophotometrically and 10-fold serially diluted with sterile water to obtain cell suspensions from 10^0 to 10^6 CFU/ml. Dilution plating was used to determine that 0.1 unit at an optical density of 600 nm equals UW551-rif at 2×10^8 CFU/ml and K60-rif at 1×10^8 CFU/ml. Serially diluted bacterial suspensions were subjected to various detection methods as described below to

Table 1. Bacterial strains, plants, and primers used in this study

Sample	Description ^a	Source
Bacteria		
K60-rif	Spontaneous rifampicin-resistant strain of <i>Ralstonia solanacearum</i> K60 (phylotype II, sequevar 7)	52
UW551-rif	Spontaneous rifampicin-resistant strain of <i>R. solanacearum</i> UW551 (phylotype II, sequevar 1)	52
Plants		
Geranium	Designer Dark Red	...
Tomato	Bonny Best	...
Tomato	BHN699 naturally infected with native phylotype II, sequevar 7 <i>R. solanacearum</i> from fields in Painter, VA	...
Potato	Superior	...
Primers		
759	5' GTCGCCGTCACCTCACTTTCC 3'	36
760	5' GTCGCCGTCAGCAATGCGGAATCG 3'	36
630	5' ATACAGAATTCGACC GGCAC 3'	11
631	5' AATCACATGCAATTCGCTAC 3'	11
rk2208.1 F3	5' GAGAGACATGTCCGATTCCG 3'	26
rk2208.1 B3	5' GCCGATGTCATCAAGCTCAA 3'	26
rk2208.1FIP	5' TGTGACTTCCACGTCAAGCGTTGCAATCACCGACTTCCTCA 3'	26
rk2208.1BIP	5' GCGAGAAGCCCGTGTGCTTGTACACGATTTCGGCCAGTT 3'	26
rk2208.1loop F	5' AGAGCTTTTCGCCAATCGACT 3'	26
rk2208.1F strand	FAM/5'ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGAAGAGCTTTTCGCCAATCGACT3'	25
Q strand	5' TCGGCATCCGCATCCGCATTTCGCATCCGGGTCCTCAGCGT 3'/BHQ	25
RRSL2403F	5' TTGGCACGAGTCTGCACTAC 3'	26
RRSL2403R	5' CACATCAGGCGATACAGTGG 3'	26
<i>Rsol_fliCF</i>	5' GAACGCCAACGGTGCGAACT 3'	46
<i>Rsol_fliCR</i>	5' GGCGGCCTTCAGGGAGGTC 3'	46

^a FAM = 6-carboxyfluorescein and BHQ = black hole quencher.

determine the detection limit of each approach. Each experiment was repeated three times (biological replicates), with two technical replicates for each cell suspension.

Dilution plating. Plant samples ground in sterile water or bacterial suspensions in water were 10-fold serially diluted and plated on CPG agar supplemented with TZC at 20 mg/ml and rifampicin at 25 mg/liter (if rifampicin-resistant strains were used). Colonies were counted after 48 h of incubation at 28°C to determine the number of culturable cells in the sample. All dilution plating experiments included three technical replicates.

Detection limits. For each tested method, the sensitivity or detection limit was reported as the bacterial population size corresponding to the most dilute sample that gave a positive result (e.g., a visible PCR band). The number of viable *R. solanacearum* cells in each sample was given as the mean (plus or minus standard error) as determined by serial dilution plating from a total of three biological replicates that each included two technical replicates.

Geranium inoculation and sampling. Ten-week-old geranium plants ('Designer Dark Red') were petiole inoculated with each of the two strains, as previously described (45). Briefly, a suspension of either 2.5×10^3 cells (K60-rif) or 2.5×10^4 cells (UW551-rif) was injected into a cut leaf petiole near the base of the plant. Plants were incubated at 24 and 19°C (UW551-rif) or 28°C (K60-rif) with a 12-h cycle of light and darkness. At 21 days postinoculation, approximately 1 cm of stem tissue was sampled from around the inoculated site from each plant. Each sample was then divided into 100-mg pieces and ground in either water or BEB1 extraction buffer (Agdia Inc., Elkhart, IN) or enriched in 5 ml of modified semiselective SMSA (Selective Medium - South Africa) (10) for 48 h at 28°C. Bacterial population sizes in geranium were determined by dilution plating and then grouped into two categories: high population ($>10^5$ CFU/g of tissue) and low population ($<10^5$ CFU/g of tissue, including plants with no bacteria detected by plating). The experiment included two biological replicates, each containing 15 geranium plants for each treatment. Population size data from infected geranium plants were analyzed using a nominal logistic model employing a χ^2 test to compare each method.

Tomato field samples. We collected symptomatic and asymptomatic tomato plants (total of 51 plants) from two fields in Painter, VA that were naturally infested with a *R. solanacearum* phylotype II, sequevar 7 strain that is closely related to K60 (61). Stem tissue (0.1 g) was ground and 10-fold serial dilutions were plated on SMSA agar to quantify *R. solanacearum* population sizes in each stem. Additionally, the cut stem from each plant was pressed onto FTA Classic Cards (GE Healthcare Life Sciences, Pittsburgh; see below) according to the manufacturer's instructions. Stem samples and FTA cards were sent to our laboratory at University of Wisconsin-Madison for further diagnosis. There, tomato stems were surfaced sterilized with 70% ethanol (sprayed with ethanol and flamed briefly) and 0.3 g of each stem sample was ground in 1 ml of sterile water. Homogenate (1 μ l) was used as template for direct PCR, as described below. An additional 0.3 g of tomato stem was enriched in 5 ml of SMSA broth for 48 h at 28°C and tested for the presence of *R. solanacearum* with Rs ImmunoStrips (Agdia, Inc.) according to the manufacturer's protocol.

PCR. PCR was performed using 12.5 μ l of GoTaq Green PCR master mix (Promega Corp., Madison, WI), hereafter referred to as GoTaq PCR, and 10 pmol of either the *R. solanacearum* universal primer pair 759/760 (36) or the R3bv2-specific primer pairs 630/631 (36) or RRS12403F/R (hereafter called 2403F/R) (26). Each sample (1 μ l; either bacterial suspension in water, SMSA-enriched sample, or plant homogenate in water) was used as template in a 25- μ l reaction, using the PCR program 96°C for 3 min; 35 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 30 s; followed by 72°C for 5 min for 759/760 and 630/631 primers. The PCR program for 2403F/R was 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 30 s (26). Bacterial suspensions of *R. solanacearum* at 10^9 CFU/ml and sterile water were used as the positive and negative control, respectively. PCR products were examined by electrophoresis on 1.5% agarose gels stained with ethidium bromide.

A positive reaction yielded a 306-bp product with the R3bv2-specific 630/631 primers, a 226-bp product with the R3bv2-specific 2403F/R primers (26), and a 280-bp product with the 759/760 *R. solanacearum* universal primers (36).

Primer pairs 759/760 and 630/631 were generally used in this study but we also determined the sensitivity of several other primer sets on UW551-rif. Bacterial suspensions containing from 10^0 to 10^5 CFU/ml were spotted on FTA cards (see below), and two 2-mm discs were used as template for GoTaq PCR with the 630/631, 759/760, and 2403F/R primers, as described above. For the Rsol_759/760 primers, a two-step PCR was used: 94°C for 5 min followed by 35 cycles at 94°C for 45 s and 68°C for 60 s, with no final extension and an expected product size of 400 bp (30,46). The PCR products were analyzed by electrophoresis in 1% agarose gels. All PCR experiments were repeated at least three times, with two technical replicates per biological replicate.

Enrichment coupled with PCR or Rs ImmunoStrip. For enrichment culture, 1 ml of bacterial suspension or 0.1 g of plant tissue was cultured in 5 ml of semiselective SMSA medium, with rifampicin at 25 mg/liter if rifampicin-resistant strains were used (10), at 28°C with 250 rpm shaking. After 24 and 48 h, the enriched culture was used either as template for PCR using the *R. solanacearum* 759/760 universal primers for samples containing strain K60-rif or the R3bv2-specific 630/631 and 2403F/R primers for samples containing strain UW551-rif, or for detection by Rs ImmunoStrips (Agdia, Inc.) according to the manufacturer's protocol. The enrichment experiments were conducted three times, with two technical replicates per biological replicate.

LAMP. The LAMP primers are specific for R3bv2; therefore, this assay was only conducted on samples containing strain UW551-rif. The LAMP reactions were prepared in a 25- μ l reaction, as described (25–27), using the same samples as for FTA cards, enrichment, and PCR. Work areas and pipettes were decontaminated with DNA-Away (Molecular Bio-Products, Inc. San Diego, CA) to avoid cross contamination. Then, 20 μ l of the master-mix solution was distributed into individual 0.2-ml MicroAmp strip tubes (Life Technologies, Grand Island NY). Sample (5 μ l) was added as template. Water and 100 ng of UW551-rif genomic DNA were used as negative and positive controls, respectively. The reaction mix was incubated at 65°C for 60 min in a real-time PCR machine (Applied Biosystems, Wilmington, MA) and the reaction was terminated by denaturing the *Bst* DNA polymerase at 80°C for 10 min. The LAMP experiments were conducted three times, with two technical replicates per biological replicate.

Kapa 3G Plant PCR kit. To determine the sensitivity of Kapa3G plant polymerase, hereafter referred to as Kapa3G, (Kapa Biosystems, Wilmington, MA) for detection of *R. solanacearum* in the presence of geranium tissue, we used a Powerlyzer (MoBio, Carlsbad, CA) to homogenize 100 mg of geranium tissue in 1 ml of each bacterial suspension (10^5 to 10^0 CFU/ml), with two cycles of 2,500 rpm for 1.5 min separated by a 4-min break for cooling. For PCR, 2 μ l of the homogenate was used as template in a 50- μ l reaction, with the following PCR program: 95°C for 10 min; 40 cycles of 95°C for 20 s, 58°C for 15 s, and 72°C for 30 s; and 72°C for 3 min. Simultaneously, 100 μ l of homogenate was spotted on an FTA card and dried at room temperature overnight. Two 2-mm discs were taken from each card using the Harris 2-mm micropunch (Electron Microscopy Sciences, Hatfield, PA) and were washed and purified following the manufacturer's procedure, as described below. Extracted DNA was used as template for PCR with the Kapa3G PCR Kit, as described above. The experiment included three biological replicates, each with two technical replicates.

FTA cards. To determine the sensitivity of the PCR assays using samples immobilized on FTA cards, we applied 100 μ l of bacterial suspension of either K60-rif or UW551-rif in sterile water to FTA Classic cards. The cards were dried at room temperature overnight and processed the next day as follows: two 2-mm discs were washed twice for 5 min each with 200 μ l of FTA purification reagent (GE Healthcare Life Sciences) and twice for 5 min each with 200 μ l of 0.1 $\times$ Tris-EDTA (10 mM Tris-HCl and 0.1 mM EDTA, pH 8.0), then

dried for at least 2 h at room temperature and used directly as template for a PCR assay with the universal or R3bv2-specific primers.

Alternatively, FTA Elute cards were used in place of FTA Classic cards to quantify bacteria in samples. For samples containing 10% plant extract, 100 mg of stem tissue from 4-week-old tomato ('Bonny Best') was homogenized in 1 ml of bacterial suspension to generate final bacterial suspensions of 10^0 to 10^6 CFU/ml. This homogenate (40 μ l) was dropped on an FTA Elute card and dried overnight at room temperature. Two 2-mm discs were vortexed three times each for 5 s in 500 μ l of sterile water, and then transferred to a 0.2-ml microcentrifuge tube containing 30 μ l of sterile water. The tube was heated to 95°C for 15 min, then briefly pulse vortexed and centrifuged to separate the matrix from the eluate, which contained the purified DNA. The eluate (5 μ l) was added as template for a real-time PCR with the 2403F/R primers using an Applied Biosystems 7300 Real-Time System, as previously described (14).

We modified the commercial FTA card method in an attempt to eliminate the potent PCR inhibitors present in geranium tissue. Briefly, 100 mg of healthy geranium stem was ground in 1 ml of water or BEB1 sampling buffer (Agdia, Inc.) containing 10^9 CFU of UW551-rif. The discs from FTA cards were heated for 10 min at 96°C after the first washing step, as described above. Two washed discs or 1 μ l of the homogenate were used as template for a PCR with the 630/631 or 2403F/R primers. In an additional treatment, the PCR mix was supplemented with 0.08 mM MgSO₄ to compensate for cofactors potentially chelated by geranium inhibitors, because divalent cation cofactors are essential for the activity of *Taq* DNA polymerase.

Tomato (Bonny Best) and potato tissue (Superior) were subjected to the same procedure to compare the effect of different plant tissues on PCR from FTA cards. Bacterial suspensions (10^9 CFU/ml) and sterile water were used as positive and negative controls, respectively. These experiments were conducted three times, with two technical replicates per biological replicate.

Testing viability of *R. solanacearum* on FTA cards. To evaluate *R. solanacearum* viability on FTA cards, we applied 100 μ l of bacterial suspension containing strain UW551-rif at 10^0 to 10^9 CFU/ml in sterile water to either FTA cards or Whatman number 1 filter paper, which were then dried for 1 h at room temperature. Cell suspensions (1 ml) or five discs from each FTA card or filter paper were enriched in 5 ml of SMSA broth for 48 h at 28°C and monitored for microbial growth, detected as visible turbidity of the broth. Turbid tubes were tested for *R. solanacearum* with Rs ImmunoStrips, according to the manufacturer's instructions.

To measure survival of R3bv2 on FTA cards in the realistic context of an infected plant sample, tomato plants (Bonny Best) were inoculated with UW551-rif by soil-soak inoculation, as previously described (53). At 3 to 6 days after inoculation, approximately 100 mg of stem tissue was collected from wilted plants and homogenized in a BEB1-buffer sampling pouch (Agdia Inc.). Five plants that tested positive with Rs ImmunoStrip (and thus contained $>10^5$ CFU/g) were analyzed further by applying 100 μ l of the homogenate to an FTA card that was then dried for 1 h at room temperature. Simultaneously, stem samples from each of these plants were pressed directly onto FTA cards and dried as described above. Five 2-mm discs from each FTA card were enriched in SMSA broth for 48 h as described above. Stem tissue (100 mg) was also directly cultured in SMSA as a positive control. The FTA card survival experiments were conducted three times.

Results

Sensitivity of detection methods for *R. solanacearum* in water and in plant geranium tissue. In agreement with previous results, serial dilution plating was the most sensitive method for detecting *R. solanacearum* in water, with a detection limit of fewer than 10 CFU/ml. We observed some variation within each 10-fold dilution, especially at lower dilutions (10^2 , 10^1 , and 10^0 CFU/ml; data not shown) but, overall, the standard error was less than 10% (Table 2).

Table 2. Lowest *Ralstonia solanacearum* population detected in water and homogenized geranium tissue by various methods, presented as mean CFU/ml as determined by dilution plating of the tested bacterial suspension^a

<i>R. solanacearum</i> assay	K60-rif	UW551-rif 630/631	UW551-rif 2403F/R
In water			
Plating ^b	$1.67 \times 10^1 \pm 4.30 \times 10^0$ A	$4.17 \times 10^1 \pm 1.96 \times 10^1$ A	$1.67 \times 10^1 \pm 4.3 \times 10^0$ A
Direct PCR ^c	$2.12 \times 10^4 \pm 1.96 \times 10^1$ B	$1.55 \times 10^4 \pm 7.20 \times 10^2$ B	$1.09 \times 10^5 \pm 2.58 \times 10^4$ B
FTA + PCR ^d	$2.12 \times 10^4 \pm 1.96 \times 10^1$ B	$1.49 \times 10^5 \pm 6.92 \times 10^3$ C	$9.39 \times 10^3 \pm 7.14 \times 10^2$ C
Enrichment-STRIP 48 ^e	$5.56 \times 10^0 \pm 3.51 \times 10^0$ A	$1.64 \times 10^2 \pm 4.58 \times 10^1$ A	$1.00 \times 10^2 \pm 2.26 \times 10^1$ A
Enrichment-PCR 48 ^f	$1.67 \times 10^1 \pm 4.30 \times 10^0$ A	$4.17 \times 10^1 \pm 1.96 \times 10^1$ A	$1.67 \times 10^1 \pm 4.3 \times 10^0$ A
LAMP ^g	n/a	$4.17 \times 10^1 \pm 1.96 \times 10^1$ A	n/a
In geranium tissue ^h			
Plating	$2.78 \times 10^1 \pm 5.56 \times 10^0$ A	$2.78 \times 10^1 \pm 5.56 \times 10^0$ A	$1.67 \times 10^1 \pm 4.3 \times 10^0$ A
Direct PCR	ND	ND	ND
FTA + PCR	ND	ND	ND
bFTA + PCR ⁱ	$2.12 \times 10^4 \pm 1.60 \times 10^3$ B	$1.72 \times 10^4 \pm 8.85 \times 10^2$ B	$9.39 \times 10^3 \pm 7.14 \times 10^2$ B
LAMP	n/a	$2.78 \times 10^1 \pm 5.56 \times 10^0$ A	n/a
Kapa3G ^j	$2.12 \times 10^4 \pm 1.60 \times 10^3$ B	$1.72 \times 10^4 \pm 8.85 \times 10^2$ B	$9.39 \times 10^3 \pm 7.14 \times 10^2$ B
FTA + Kapa3G ^k	$2.12 \times 10^4 \pm 1.60 \times 10^3$ B	$1.72 \times 10^4 \pm 8.85 \times 10^2$ B	$9.39 \times 10^3 \pm 7.14 \times 10^2$ B

^a Bacterial population mean values followed by different uppercase letters are significantly different from others in the same column (analysis of variance, $P < 0.05$). n/a = not available; ND = not detected, even in the positive control.

^b Plating = serial dilution plating on casamino acid peptone glucose medium.

^c Direct PCR = polymerase chain reaction (PCR) with GoTaq using the 759/760 *R. solanacearum* universal primers (for K60-rif) and the 630/631 or 2403F/R primers (for UW551-rif).

^d FTA + PCR = GoTaq PCR directly from FTA cards spotted with bacterial suspension using the 759/760 *R. solanacearum* universal primers (for K60-rif); and the 630/631 or 2403F/R primers (for UW551-rif).

^e Enrichment-STRIP 48: enrichment culture for 48 h followed by Rs ImmunoStrip.

^f Enrichment-PCR 48: enrichment culture for 48 h followed by GoTaq PCR, using the 759/760 *R. solanacearum* universal primers (for K60-rif); and the 630/631 or 2403F/R primers (for UW551-rif).

^g LAMP = loop-mediated isothermal amplification.

^h Bacteria were serially diluted in a solution of 0.1 g/ml of geranium tissue homogenized in water.

ⁱ bFTA + PCR = GoTaq PCR from FTA cards spotted with bacterial suspension, following buffer and heat treatment, using the 759/760 *R. solanacearum* universal primers (for K60-rif) and the 630/631 or 2403F/R primers (for UW551-rif).

^j Kapa3G = PCR with Kapa3G plant polymerase, using the 759/760 *R. solanacearum* universal primers (for K60-rif) and the 630/631 or 2403F/R primers (for UW551-rif).

^k FTA + Kapa3G = PCR with Kapa3G plant polymerase from FTA cards spotted with bacterial suspension, using the 759/760 *R. solanacearum* universal primers (for K60-rif) and the 630/631 or 2403F/R primers (for UW551-rif).

Direct conventional *Taq* PCR analysis of bacterial suspensions was much less sensitive than plating, with a detection limit of 10^4 CFU/ml for K60-rif when the 759/760 *R. solanacearum* universal primers were used. Because several different primer pairs have been developed for detection of *R. solanacearum*, we compared the sensitivity of four primer sets: 759/760 and *fliCF/R*, which detect all *R. solanacearum* strains, and 630/631 and 2403F/R, which are specific for R3bv2 strains. The first three primer sets had a detection limit of approximately 10^5 CFU but 2403F/R was more sensitive under these conditions by an order of magnitude, detecting 10^4 CFU/ml (Supplementary Fig. S1).

Enrichment in SMSA broth followed by either ImmunoStrip or PCR was highly sensitive, with a detection limit of approximately 10 cells/ml for K60-rif (Table 2). Enrichment plus PCR was similarly sensitive for UW551-rif and K60-rif but enrichment plus ImmunoStrip was not as effective for UW551-rif, indicating that this method's detection limit varied by strain. This difference could result from strain-to-strain variation in growth rate during enrichment or in production of extracellular polysaccharide, which contains the epitope recognized by the antibody in the ImmunoStrips.

Conducting GoTaq PCR directly on samples immobilized on FTA cards yielded a sensitivity of 10^4 CFU/ml for both K60-rif (using the 759/760 primers) and UW551-rif (with the 2403F/R primers), but this method was less sensitive for UW551-rif with 630/631 primers (10^5 CFU/ml; Table 2). The LAMP assay had a detection limit of 4.17×10^1 CFU/ml, making it as sensitive as enrichment followed by PCR for the detection of UW551-rif cells in water.

However, when bacterial suspensions were combined with geranium tissue, conventional PCR with GoTaq polymerase failed to detect *R. solanacearum*, even when the bacterial population size exceeded 10^9 CFU/ml (Table 2; Fig. 1). PCR amplification was also completely inhibited for bacterial suspensions containing geranium tissue that were applied to FTA cards and tested using conventional GoTaq polymerase, even after multiple washing steps. Thus, conventional PCR was ineffective for samples containing geranium tissue. In contrast, potato tuber and tomato stem tissue did not inhibit PCR amplification from homogenized samples (not shown) or from samples on FTA cards (Fig. 1).

The LAMP assay detected *R. solanacearum* R3bv2 in the presence of geranium inhibitors, with sensitivity as low as 10^1 CFU/ml. However, LAMP was prone to false positives in this experiment. Persistent problems with reproducibility indicated that the LAMP assay may be too technically demanding for routine diagnostic use.

Fig. 1. Detection of *Ralstonia solanacearum* UW551-rif by polymerase chain reaction (PCR) directly from plant tissue or after sample application onto FTA cards. Plant tissue (geranium, potato, or tomato) was homogenized in water or Agdia BEB1 buffer containing 10^9 cells of UW551-rif and either boiled or used directly as template for PCR using the 759/760 *R. solanacearum* universal primers. The homogenate was also applied onto FTA Classic cards and dried overnight before PCR. Two 2-mm punches from each card were washed and used as template in a 25- μ l PCR. The experiment was repeated three times.

Viability of *R. solanacearum* on FTA cards. To determine whether *R. solanacearum* R3bv2 could survive on FTA cards, we used the enrichment method to detect viable cells 1 h after application of cell suspensions containing UW551-rif at 10^9 to 10^0 CFU/ml to FTA cards or Whatman number 1 filter paper. After 48 h of enrichment in SMSA at 28°C, tubes containing five 2-mm discs from filter paper spotted with 10^9 to 10^5 CFU/ml became turbid and tested positive with Rs ImmunoStrips. In contrast, none of the cultured FTA-card discs were turbid or tested positive with the ImmunoStrips after 48 h. When water suspensions of bacterial cells were enriched in SMSA, the limit of detection was 10^0 CFU/ml (Supplementary Table S1).

To determine whether plant material enhanced survival of bacteria on FTA cards, we tested the viability of bacteria derived from tomato plants inoculated with R3bv2 strain UW551-rif following immobilization on FTA cards. Stem samples from plants that tested positive with Agdia Rs ImmunoStrips (and thus harbored at least 1×10^5 CFU/g of tissue) were pressed on either FTA cards or filter paper and cultured in SMSA at 28°C. After 48 h of enrichment, all tubes containing discs from filter paper became turbid and tested positive for *R. solanacearum* with ImmunoStrips. In contrast, no microbial growth was observed following culture of any FTA-card discs from infected plants (Supplementary Fig. S2).

Optimization of the FTA card protocol for plant samples. Adding the *Taq* DNA polymerase cofactor Mg^{2+} did not relieve PCR inhibition by geranium tissue, suggesting that the inhibitors present in geranium do not act by chelation of divalent cations (39). However, including heat treatment in the presence of extraction buffer resulted in successful amplification of UW551 DNA from FTA cards in the presence of geranium tissue (Fig. 1). This treatment yielded a detection limit of approximately 10^4 CFU/ml for both K60-rif and UW551-rif cell suspension in the presence of geranium tissue extracts (Table 2). The detection limit of quantitative PCR on DNA extracted from FTA Elute cards was only 10^5 CFU/ml of homogenate.

Kapa3G improved amplification of *R. solanacearum* DNA in the presence of geranium tissue, either directly from bacterial suspensions plus geranium homogenate or following application of bacterial cells plus geranium homogenate to FTA cards (Fig. 1). Conducting Kapa3G PCR on serial dilutions of K60-rif and UW551-rif cell suspensions with geranium tissue resulted in a detection limit of approximately 10^4 CFU/ml (Table 2).

Detection of *R. solanacearum* in infected geranium plants. Enrichment for 48 h followed by ImmunoStrip detected *R. solanacearum* in all inoculated samples but none of the uninoculated negative controls (Table 3). This confirmed that all the tested geranium plants were infected. The longer incubation period was needed for optimal sensitivity, because after only 24 h of enrichment neither ImmunoStrip nor PCR detected *R. solanacearum* in 100% of the samples.

Plants contained variable bacterial population sizes 3 weeks after inoculation, as revealed by dilution plating. About two-thirds of the geranium contained $>10^5$ CFU/g stem and were classified as "high" populations, while the remainder contained $<10^5$ CFU/g stem and were classified as "low" (including plants with no detectable bacteria by dilution plating). This variation allowed us to compare the sensitivity of detection methods on plants with a range of colonization levels. On infected geranium plants, the detection methods gave similar results for both K60 and UW551, with the exception of the LAMP assay, which was tested only on plants infected with UW551.

Most detection methods performed well on infected geranium plants that contained high bacterial populations ($>10^5$ CFU/g of crown tissue). Exceptions were direct PCR from plant tissue and PCR from plant samples applied to FTA cards, which were completely ineffective, presumably because of the PCR inhibitors in geranium tissue. The tested detection methods fell into four partially overlapping significance groups (Table 3). The first group included the ineffective PCR-based methods and a second group contained tests of intermediate sensitivity, including ImmunoStrip, enrichment culture for either 24 or 48 h followed by PCR, and LAMP. The most effective group of detection methods included dilution plating, enrichment culture for either 24 or 48 h followed by ImmunoStrip

testing, and PCR from plant samples applied to FTA cards and then boiled.

For plants containing low bacterial populations ($<10^5$ CFU/g of crown tissue), the tested detection methods fell into three nonoverlapping significance groups (Table 3). As observed for plants containing high bacterial populations, neither direct PCR from plant tissue nor PCR from plant samples applied to FTA cards gave positive results. The sensitivities of enrichment culture for 24 h followed by PCR, enrichment culture for 48 h followed by PCR, ImmunoStrip testing, LAMP, dilution plating, and 24 h of enrichment followed by ImmunoStrip testing were statistically indistinguishable ($P < 0.01$). The most effective group of methods included PCR from plant samples applied to FTA cards and boiled, and 48 h of enrichment culture followed by ImmunoStrip testing ($P < 0.01$). However, only 48 h of enrichment culture followed by ImmunoStrip testing detected all geranium plants containing $<10^5$ CFU/g.

Even though the detection limit of the LAMP assay was $<10^2$ CFU/ml with water suspensions of *R. solanacearum* with or without added geranium tissue, this assay performed poorly on infected geranium samples. LAMP detected only 75 and 95% of the poorly and highly colonized plants, respectively. Thus, this assay gave between 25 and 5% false-negative results on R3bv2-infected geranium plants.

Detection of *R. solanacearum* in tomato field samples. Because tomato tissue did not inhibit PCR, no further optimization of PCR was needed. We grouped the samples by the presence of symptoms. Plant samples that yielded typical *R. solanacearum* colonies on SMSA medium were classed as “population size known”, while samples yielding no likely *R. solanacearum* colonies were categorized as “population size unknown”.

All tested methods gave uniformly positive results on samples from field-grown symptomatic plants and from asymptomatic plants containing detectable populations of *R. solanacearum*. Asymptomatic

samples with unknown *R. solanacearum* populations also had high detection rates of 94.4, 94.4, and 88.2% for direct GoTaq PCR, GoTaq PCR from FTA cards, and 48 h of enrichment followed by ImmunoStrip, respectively (Table 4).

The average bacterial population size in wilted tomato plants was higher than populations in asymptomatic plants by almost an order of magnitude. It is worth noting that the number of asymptomatic plants with undetectable bacterial populations was twice the number of asymptomatic plants with detectable populations. Only 30% of the symptomatic plants from the field contained no *R. solanacearum* detectable by dilution plating.

Practical considerations of detection methods. We evaluated practical aspects of the methods used in this study by estimating the time, cost, and complexity of each method. The cost of diagnosis included only the cost of reagents in 2013 dollars, without equipment or labor expenses. Time for each method was from sample preparation to final result, in hours. We also rated the complexity of each method by the following scale: very easy (no special training required), easy (requires minimal microbiological experience and training), moderate (assay requires some specialized skills to achieve replicable results), and difficult (assay requires extensive experience and special handling of the samples) (Table 5).

Of the detection methods considered in this study, direct Rs ImmunoStrip was the fastest and easiest, although it was also the most expensive assay. All the culture-based approaches (plating and the two enrichment culture methods) required a 24- to 48-h incubation period. Although the two enrichment assays were easy to perform, their prices varied widely from \$6.25/sample for enrichment plus ImmunoStrip to \$0.47/sample for enrichment plus PCR. For FTA cards followed by PCR, the cost was \$1.90 to 3.30, with the FTA card accounting for much of the total cost. Except for geranium, grinding samples in BEB1 buffer was not required to eliminate PCR inhibitors;

Table 3. Percentage of *Ralstonia solanacearum*-infected geranium plants detected by various methods^a

Method ^d	Low ^b		High ^c	
	K60 (<i>n</i> = 10)	UW551 (<i>n</i> = 8)	K60 (<i>n</i> = 20)	UW551 (<i>n</i> = 22)
Direct PCR	0.0 A	0.0 A	0.0 A	0.0 A
FTA card + PCR	0.0 A	0.0 A	0.0 A	0.0 A
Rs ImmunoStrip	10.0 B	12.5 B	95.0 B	86.4 B
Enrichment-PCR 24 h	10.0 B	62.5 B	90.0 BC	100.0 BC
Enrichment-PCR 48 h	20.0 B	62.5 B	95.0 BC	100.0 BC
LAMP	n/a	75.0 B	n/a	95.5 BC
Plating	30.0 B	87.5 B	100.0 C	100.0 C
Enrichment-STRIP 24 h	80.0 B	75.0 B	100.0 C	100.0 C
Boiled FTA card + PCR	90.0 C	87.5 C	100.0 C	86.4 C
Enrichment-STRIP 48 h	100.0 C	100.0 C	100.0 C	100.0 C

^a Values followed by different uppercase letters are different from others in the same column ($P < 0.01$, odds ratio χ^2 tests comparing each method to every other method); n/a = not applicable, because loop-mediated isothermal amplification (LAMP) primers are specific to race 3 biovar 2 strains.

^b Low = bacterial population less than 10^5 CFU/g stem or undetected.

^c High = bacterial population higher than 10^5 CFU/g stem.

^d PCR = polymerase chain reaction; Enrichment-PCR = plant tissues were cultured in liquid SMSA for 24 or 48 h, followed by GoTaq-PCR using the 759/760 *R. solanacearum* universal primers (for K60-rif), and the 630/631 primers (for UW551-rif); and Enrichment-STRIP = plant tissues were cultured in liquid SMSA for 24 or 48 h, followed by Rs ImmunoStrip.

Table 4. Percentage of tomato field samples from Virginia testing positive for *Ralstonia solanacearum* with various methods^a

Sample, bacterial population size	N ^b	PCR ^c	FTA + PCR ^d	BIO-PCR 48 ^e
Asymptomatic plants				
Size known ($\bar{x} = 7.54 \times 10^9 \pm 6.23 \times 10^9$ CFU/g)	9	100 $\pm$ 0 A	100 $\pm$ 0 A	100 $\pm$ 0 A
Size unknown	18	95.46 $\pm$ 4.55 A	95.46 $\pm$ 4.55 A	85.72 $\pm$ 14.29 A
Symptomatic plants				
Size known ($\bar{x} = 6.59 \times 10^{10} \pm 1.22 \times 10^{10}$ CFU/g)	15	100 $\pm$ 0 A	100 $\pm$ 0 A	100 $\pm$ 0 A
Size unknown	9	100 $\pm$ 0 A	100 $\pm$ 0 A	100 $\pm$ 0 A

^a Values followed by different uppercase letters are significantly different from others in the same column (analysis of variance, $P < 0.05$).

^b Sample size.

^c GoTaq polymerase chain reaction (PCR) from ground tomato stem sections using the 759/760 *R. solanacearum* universal primers.

^d FTA + PCR = GoTaq PCR from tomato tissue immobilized on FTA cards using the 759/760 *R. solanacearum* universal primers.

^e BIO-PCR 48 = enrichment culture of ground tomato stem sections for 48 h followed by testing with Rs ImmunoStrip.

therefore, the cost of an assay for nongeranium hosts could be reduced by one-third by applying plant samples directly to FTA cards. However, ImmunoStrip testing in the field requires sampling pouches that already contain BEB1 buffer; thus, FTA cards can be used on the same samples with no extra cost.

Direct PCR from plant samples was also rapid and inexpensive but there is a high risk of false-negative results because of PCR inhibitors, especially with geranium tissue. LAMP was the most complex assay, because it required expertise to avoid false positives from cross-contamination. Although, under ideal conditions, the LAMP assay was quick, specific to *R. solanacearum* R3bv2, and tolerant to PCR inhibitors, it also gave some false-negative results with infected geranium samples. The Kapa3G PCR kit, alone or coupled with FTA cards, took only a few minutes longer than conventional PCR, specifically detected R3bv2, and cost about \$1.05 to \$2.95 per sample. This was more expensive than direct PCR with conventional polymerase but cheaper than PCR from FTA cards with the sample homogenized in BEB1 buffer. More importantly, PCR using Kapa3G on samples immobilized on FTA cards could simultaneously overcome the PCR inhibitors in geranium and allow secure transport of suspect plant materials.

Discussion

Following passage of the U.S. PATRIOT Act in 2001, there was a significant reduction in efficiency of research involving Select Agents (8). In an effort to facilitate Select Agent research without compromising biosecurity, we developed a procedure for sensitive detection of *R. solanacearum* in plant samples, especially the Select Agent R3bv2 strains. We compared this new protocol to several existing diagnostic assays in terms of detection limit and feasibility (time, cost, and difficulty).

Detecting the bacterium in water was relatively straightforward, although the sensitivity varied among methods and strains. Dilution plating and enrichment coupled to PCR gave the most consistent results for both strains, with a detection limit of approximately 10^1 CFU/ml. This is consistent with previous studies using these methods (10,30). A very low detection limit of 10^0 to 10^2 was previously reported for PCR following enrichment (30). Culture-based methods have the advantage of detecting viable cells from small populations,

which is critical for screening latently infected plants that may harbor fewer than 10^4 CFU/g of tissue (7,10).

Inhibitory compounds in plant or environmental samples pose a major obstacle to nucleic acid-based diagnosis of plant pathogens (12,18,32,60,62). Various expensive and time-consuming protocols have been developed to remove or inactivate potential PCR inhibitors, including addition of PCR adjuvants (33,62), filtration (27,52), or immunomagnetic separation (14). To address this problem, we used FTA cards, which were developed to immobilize and preserve DNA from biological samples (1,6,13,43). However, the FTA cards still contained PCR inhibitors from residual geranium tissue, requiring additional treatment with a sampling buffer and heat. A similar protocol was reported to remove geranium inhibitors using BEB1 buffer and microwave combined with the GenRelease kit; however, the low DNA yield of the kit reduced the detection limit in poorly colonized tissues (12). Amplifying DNA with the experimentally evolved *Taq* polymerase Kapa3G also eliminated PCR inhibition by plant tissue. This approach had a detection limit comparable with that of the modified FTA card protocol (10^4 CFU/ml), so that either method could be used when PCR inhibition is a problem. However, this product has been evaluated on a limited number of plant species (22) and *R. solanacearum* has a wide host range; therefore, the efficiency of Kapa3G should be tested on samples from other hosts.

Plant samples are often shipped via mail or carried by researchers from the field to the lab. FTA card technology allows easy storage and secure transport of samples because FTA cards contain reagents that immediately lyse cells and release their DNA, which becomes stably affixed to the card matrix and available for PCR amplification. A previous study showed that over 100 bacterial strains did not survive on FTA cards, with the exception of some spore-forming bacilli that remained viable only at high cell densities (43). We showed that *R. solanacearum* R3bv2 could not survive on the FTA cards with or without the presence of plant material, even at high cell densities. Thus, FTA cards can be used to store *R. solanacearum* R3bv2 DNA from infected plant materials and safely transport it to diagnostic laboratories without compromising agricultural biosecurity.

The FTA Elute card followed by real-time PCR allowed quantification of bacterial populations in geranium tissue with a detection

Table 5. Practical considerations for various methods for detection of *Ralstonia solanacearum*

Method	Total time (h) ^a	Hands-on time ^b	Cost (USD) ^c	Complexity ^d
Enrichment-PCR 48 ^e	50.5	3.0	\$ 0.47	***
Enrichment-STRIP 48 ^f	48.5	1.0	\$ 6.25	**
Plating ^g	48.0	0.5	\$ 0.26	***
Enrichment-PCR 24	26.5	3.0	\$ 0.47	***
Enrichment-STRIP 24	24.5	1.0	\$ 6.25	**
FTA + PCR ^h	3.0	1.0	\$ 1.90	***
bFTA + PCR ⁱ	3.0	2.0	\$ 3.30	***
FTA + Kapa3G ^j	3.0	1.5	\$ 2.95	**
Direct PCR ^k	2.5	0.5	\$ 0.42	***
Kapa3G ^l	2.5	0.5	\$ 1.05	**
LAMP ^m	1.5	0.5	\$ 2.51	****
Rs ImmunoStrip ⁿ	0.5	0.5	\$ 6.20	*

^a Total time to complete an assay (from sample preparation to obtaining result).

^b Time required for direct handling.

^c In 2013 U.S. dollars. Cost represents reagent expense for each method. Labor and equipment cost were not included.

^d Complexity: * = very easy (requires no training, assay is easy and highly replicable), ** = easy (requires minimal microbiological expertise and training to achieve replicable results), *** = moderate (requires some technical skill to achieve replicable results), **** = difficult (requires extensive experience to achieve replicable results)

^e Enrichment culture for 24 or 48 h followed by polymerase chain reaction (PCR).

^f Enrichment culture for 24 or 48 h followed by Rs ImmunoStrip.

^g Serial dilution plating.

^h FTA + PCR = GoTaq PCR from FTA cards spotted with bacterial suspension.

ⁱ bFTA + PCR = Buffer- and heat-treated samples on FTA card followed by GoTaq PCR.

^j FTA + Kapa3G = PCR from samples on FTA cards using Kapa3G.

^k Direct PCR = PCR with GoTaq.

^l Kapa3G = PCR with Kapa3G plant polymerase.

^m Loop-mediated isothermal amplification.

ⁿ Rs ImmunoStrip = samples tested directly with the Agdia Rs ImmunoStrip lateral flow device.

limit of 10^5 CFU/ml (which corresponds to approximately 5×10^3 cells per PCR), in agreement with results obtained with potato extract (14). Although the same sample volume was used in a real-time reaction, the actual amount of bacterial DNA in 5 μ l of eluate from our FTA card was less than the volume of potato extract used in the previous study (14). Therefore, this method did not appear practical for quantification of *R. solanacearum* in plant tissue, unless more FTA discs or more eluate are used to improve the sensitivity.

Asymptomatic but latently infected geranium plants can contain *R. solanacearum* populations between 10^4 and 10^8 CFU/g. We found that enrichment-based detection methods could sensitively detect the presence of *R. solanacearum*, but only when samples were incubated for 48 h. The lower detection sensitivity of enrichment coupled with PCR was most likely due to residual geranium PCR inhibitors in the enrichment broth because, in water, this method detected 10^1 CFU/ml. Using enrichment coupled with PCR or enrichment followed by ImmunoStrip, we confirmed that no inoculated geranium plants experienced infection.

Although theoretically promising (28), the LAMP assay did not perform well in our hands. LAMP was sensitive for R3bv2 cell suspension in water, detecting as little as 10^1 CFU/ml, but this method yielded between 5 and 25% false negatives when it was used on infected geranium plants. Because of its high sensitivity, LAMP requires extreme care to avoid false positives caused by cross-contamination (25), which were a consistent problem in spiked samples. A false-positive diagnosis could cause unnecessary economic loss for growers, while a false-negative diagnosis could lead to an incorrect quarantine decision. The potential for these two significant errors limits the usefulness of this assay.

Unlike geranium plants, which present ambiguous symptoms and are often latently infected, tomato plants with bacterial wilt are easy to diagnose. Diseased plants present a distinctive combination of symptoms (stunting, wilting, and vascular browning) and signs (bacterial streaming from cut stems). Most tomato varieties are susceptible to bacterial wilt, and even asymptomatic infected plants generally contain large bacterial populations ($>10^5$ CFU/g stem). However, because of the current “guilty until proven innocent” Select Agent regulations (55), plant pathologists and breeders in the United States need a diagnostic method that can rapidly and securely distinguish between endemic strains of *R. solanacearum* and R3bv2 strains. Using naturally infected plants from a Virginia tomato field, we showed that *R. solanacearum* DNA immobilized on FTA cards could be successfully amplified by PCR without further processing. We propose that PCR amplification using the *R. solanacearum* universal primer pair 759/760 coupled with R3bv2-specific primer pair 2403F/R or 630/631 could reliably and safely identify endemic North American bacterial wilt strains and simultaneously show that they are not R3bv2. Such strains could then be exempted from the Select Agent regulations, allowing unencumbered research on bacterial wilt management strategies for American tomato growers.

Fig. 2. Proposed pipeline flowchart for sampling and detecting *Ralstonia solanacearum* R3bv2 in plant samples using FTA cards.

Colonization of *R. solanacearum* in field-grown tomato plants was heterogeneous, as previously observed (48). All symptomatic plants tested positive with every assay used, although the bacterial population in stems (as detected by dilution plating) varied among plants, possibly because *R. solanacearum* was out-competed or inhibited by fast-growing saprophytic microbes. Thus, wilting symptoms of tomato are not invariably associated with an *R. solanacearum* population that can be easily detected by culture-based methods. Although enrichment followed by ImmunoStrip was sensitive for detecting *R. solanacearum* cells in water, this method was less sensitive than either direct PCR and FTA card plus PCR on poorly colonized or highly contaminated field tomato samples. SMSA is only a semiselective medium, and turbid enrichment cultures from field tomato samples sometimes did not contain *R. solanacearum* as determined by conventional PCR, even though stem homogenates from those same samples gave a positive result with direct PCR or enrichment plus ImmunoStrip (data not shown). This may be explained by large populations of other microbes that reduce PCR efficiency (47). Alternatively, growth of small initial *R. solanacearum* populations might have been inhibited by field microbes during enrichment (7,48). Therefore, incubation time is a critical variable for diagnosis of field samples because a long enrichment period can allow growth of non-target organisms that reduce assay sensitivity.

Despite becoming more rapid, sensitive, and affordable, nucleic acid-based techniques are not widely used for field diagnosis of plant diseases because most of them require equipment available only in laboratories (32). Moreover, Select Agents such as *R. solanacearum* R3bv2 cannot be easily tested by PCR because biosecurity regulations constrain the shipment of samples to diagnostic laboratories from remote locations. Currently, any wilting geranium plant found in the United States that tests positive with the Rs ImmunoStrip must be immediately sent to the United States Department of Agriculture APHIS Plant Protection and Quarantine laboratory in Beltsville, MD to determine whether it is infected with *R. solanacearum* R3bv2. Off-shore producers generally have no way to determine whether a wilting geranium plant that tests positive with an ImmunoStrip is infected with an R3bv2 strain of *R. solanacearum*. The methods presented here would allow growers or researchers to safely transport plant material from production sites back to laboratories for confirmatory testing. Testing plants with a lateral flow device like the Rs ImmunoStrip requires minimal experience and can be easily done at any field or greenhouse. However, the Rs ImmunoStrip will not give a positive result on plants that contain less than 10^5 CFU/g of stem (52). Geranium samples can be pressed onto FTA cards and sent to laboratories for molecular diagnosis using PCR with Kapa3G DNA polymerase or heat and buffer treatment plus GoTaq PCR. Importantly, shipping samples on these cards is comparable with transporting cell-free DNA, because we demonstrate that *R. solanacearum* does not survive on FTA cards and would not pose any biosecurity threat.

In summary, we evaluated several detection techniques for *R. solanacearum* R3bv2 in the context of strict quarantine regulations and the potent PCR inhibitors present in geranium tissue. Based on our results, we propose a pipeline to screen large numbers of geranium plants for infection with *R. solanacearum* R3bv2 using strain-specific PCR on DNA immobilized on FTA cards (Fig. 2). We also identified a reliable, sensitive, and cost-effective method to distinguish endemic North American *R. solanacearum* strains from Select Agent R3bv2 strains on field-grown tomato plants. These methods have potential application for management of bacterial wilt diseases, because they could be adapted to monitor the pathogen's presence in soil, water, and weed hosts where *R. solanacearum* can persist and threaten crop production (2,3,19,57).

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Literature Cited

- Ahmed, H. A., MacLeod, E. T., Hide, G., Welburn, S. C., and Picozzi, K. 2011. The best practice for preparation of samples from FTA cards for diagnosis of blood borne infections using African trypanosomes as a model system. *Parasit. Vectors* 4:1-7.
- Álvarez, B., López, M. M., and Biosca, E. G. 2008. Survival strategies and pathogenicity of *Ralstonia solanacearum* phylotype II subjected to prolonged starvation in environmental water microcosms. *Microbiology* 154:3590-3598.
- Caruso, P., Palomo, J. L., Bertolini, E., Alvarez, B., Lopez, M. M., and Biosca, E. G. 2005. Seasonal variation of *Ralstonia solanacearum* biovar 2 populations in a Spanish river: Recovery of stressed cells at low temperatures. *Appl. Environ. Microbiol.* 71:140-148.
- Cellier, G., Remenant, B., Chiroleu, F., Lefeuvre, P., and Prior, P. 2012. Phylogeny and population structure of brown rot-and Moko disease-causing strains of *Ralstonia solanacearum* phylotype II. *Appl. Environ. Microbiol.* 78:2367-2375.
- Clarke, C. R., Studholme, D. J., Hayes, B., Runde, B., Weisberg, A., Cai, R., Wroblewski, T. C., Daunay, M., Wicker, E., Castillo, J. A., and Vinatzer, B. A. 2015. Genome-enabled phylogeographic investigation of the quarantine pathogen *Ralstonia solanacearum* race 3 biovar 2 and screening for sources of resistance against its core effectors. *Phytopathology* 105:597-607.
- Cox, A. P., Tosas, O., Tilley, A., Picozzi, K., Coleman, P., Hide, G., and Welburn, S. C. 2010. Constraints to estimating the prevalence of trypanosome infections in East African zebu cattle. *Parasit. Vectors* 3:1-8.
- Denny, T. 2006. Plant pathogenic *Ralstonia* species. Pages 573-644 in: *Plant-Associated Bacteria*. S. S. Gnanamanickam, ed. Springer, Dordrecht, The Netherlands.
- Dias, M. B., Reyes-Gonzalez, L., Veloso, F. M., and Casman, E. A. 2010. Effects of the USA PATRIOT Act and the 2002 Bioterrorism Preparedness Act on select agent research in the United States. *Proc. Natl. Acad. Sci. USA* 107:9556-9561.
- Elphinstone, J. 2005. The current bacterial wilt situation: A global overview. Pages 9-28 in: *Bacterial Wilt Disease and the Ralstonia solanacearum Species Complex*. C. Allen, P. Prior, and A. Hayward, eds. American Phytopathological Society, St. Paul, MN.
- Elphinstone, J., Hennessy, J., Wilson, J., and Stead, D. 1996. Sensitivity of different methods for the detection of *Ralstonia solanacearum* in potato tuber extracts. *EPPO Bull.* 26:663-678.
- Fegan, M., Holoway, G., Hayward, A., and Timmis, J. 1998. Development of a diagnostic test based on the polymerase chain reaction (PCR) to identify strains of *Ralstonia solanacearum* exhibiting the biovar 2 genotype. Pages 34-43 in: *Bacterial Wilt Disease: Molecular and Ecological Aspects*. P. Prior, C. Allen, and J. Elphinstone, eds. Springer, Berlin, New York.
- Glick, D., Coffey, C., and Sulzinski, M. 2002. Simultaneous PCR detection of the two major bacterial pathogens of geranium. *J. Phytopathol.* 150: 54-59.
- Grund, E., Darissa, O., and Adam, G. 2010. Application of FTA®Cards to sample microbial plant pathogens for PCR and RT-PCR. *J. Phytopathol.* 158:750-757.
- Ha, Y., Kim, J.-S., Denny, T. P., and Schell, M. A. 2012. A rapid, sensitive assay for *Ralstonia solanacearum* race 3 biovar 2 in plant and soil samples using magnetic beads and real-time PCR. *Plant Dis.* 96:258-264.
- Hayward, A. C. 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. *Annu. Rev. Phytopathol.* 29:65-87.
- Hayward, A. C. 1995. *Pseudomonas solanacearum*. Pages 139-151 in: *Pathogenesis and Host Specificity in Plant Diseases: Histopathological, Biochemical, Genetic, and Molecular Bases*. U. S. Singh, R. P. Singh, and K. Kohmoto, eds. Vol. 1. Pergamon, Oxford, U.K. and Tarrytown, NY.
- Hendrick, C. A., and Sequeira, L. 1984. Lipopolysaccharide-defective mutants of the wilt pathogen *Pseudomonas solanacearum*. *Appl. Environ. Microbiol.* 48:94-101.
- Henson, J. M., and French, R. C. 1993. The polymerase chain reaction and plant disease diagnosis. *Annu. Rev. Phytopathol.* 31:81-109.
- Hong, J. C., Momol, M. T., Jones, J. B., Ji, P., Olson, S. M., Allen, C., Perez, A., Pradhanang, P., and Guven, K. 2008. Detection of *Ralstonia solanacearum* in irrigation ponds and aquatic weeds associated with the ponds in North Florida. *Plant Dis.* 92:1674-1682.
- Imazaki, I., and Nakaho, K. 2010. Pyruvate-amended modified SMSA medium: Improved sensitivity for detection of *Ralstonia solanacearum*. *J. Gen. Plant Pathol.* 76:52-61.
- Janse, J., Van den Beld, H., Elphinstone, J., Simpkins, S., Tjou-Tam-Sin, N., and Van Vaerenbergh, J. 2004. Introduction to Europe of *Ralstonia solanacearum* biovar 2, race 3 in *Pelargonium zonale* cuttings. *J. Plant Pathol.* 86:147-155.
- Kapa Biosystems. 2013. Kapa3G Plant PCR Kits Brochure. Wilmington, MA.
- Kelman, A. 1954. The relationship of pathogenicity of *Pseudomonas solanacearum* to colony appearance in a tetrazolium medium. *Phytopathology* 44:693-695.
- Kim, S., Olson, T., Schaad, N., and Moorman, G. 2003. *Ralstonia solanacearum* race 3, biovar 2, the causal agent of brown rot of potato, identified in geraniums in Pennsylvania, Delaware, and Connecticut. *Plant Dis.* 87:450.
- Kubota, R., Jenkins, D., and Alvarez, A. 2011. FRET-based assimilating probe for sequence specific real-time monitoring of loop mediated isothermal amplification. *Biol. Eng. Trans.* 4:81-100.
- Kubota, R., Schell, M. A., Peckham, G. D., Rue, J., Alvarez, A. M., Allen, C., and Jenkins, D. M. 2011. In silico genomic subtraction guides development of highly accurate, DNA-based diagnostics for *Ralstonia solanacearum* race 3 biovar 2 and blood disease bacterium. *J. Gen. Plant Pathol.* 77:182-193.
- Kubota, R., Vine, B. G., Alvarez, A. M., and Jenkins, D. M. 2008. Detection of *Ralstonia solanacearum* by loop-mediated isothermal amplification. *Phytopathology* 98:1045-1051.
- Lenarčič, R., Morisset, D., Pirc, M., Llop, P., Ravnikar, M., and Dreo, T. 2014. Loop-mediated isothermal amplification of specific endoglucanase gene sequence for detection of the bacterial wilt pathogen *Ralstonia solanacearum*. *PLoS One* 9:e96027.
- Li, R., Mock, R., Huang, Q., Abad, J., Hartung, J., and Kinard, G. 2008. A reliable and inexpensive method of nucleic acid extraction for the PCR-based detection of diverse plant pathogens. *J. Virol. Methods* 154:48-55.
- Li, X., Nie, J., Hammill, D. L., Smith, D., Xu, H., and De Boer, S. H. 2014. A comprehensive comparison of assays for detection and identification of *Ralstonia solanacearum* race 3 biovar 2. *J. Appl. Microbiol.* 117:1132-1143.
- López, M., Quesada, J., Penyalver, R., Biosca, E., Caruso, P., Bertolini, E., and Llop, P. 2008. Current technologies for *Pseudomonas* spp. and *Ralstonia solanacearum* detection and molecular typing. Pages 3-19 in: *Pseudomonas syringae Pathovars and Related Pathogens—Identification, Epidemiology and Genomics*. M. Fatmi, A. Collmer, N. S. Iacobellis, J. Mansfield, J. Murillo, N. W. Schaad, and M. Ullrich, eds. D. Springer, London.
- López, M. M., Llop, P., Olmos, A., Marco-Noales, E., Cambra, M., and Bertolini, E. 2009. Are molecular tools solving the challenges posed by detection of plant pathogenic bacteria and viruses? *Curr. Issues Mol. Biol.* 11:13-46.
- Ma, Z., and Michailides, T. J. 2007. Approaches for eliminating PCR inhibitors and designing PCR primers for the detection of phytopathogenic fungi. *Crop Prot.* 26:145-161.
- Milling, A., Meng, F., Denny, T. P., and Allen, C. 2009. Interactions with hosts at cool temperatures, not cold tolerance, explain the unique epidemiology of *Ralstonia solanacearum* race 3 biovar 2. *Phytopathology* 99:1127-1134.
- Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., and Hase, T. 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.* 28:e63.
- Opina, N., Tavner, F., Hollway, G., Wang, J., Li, T., Maghirang, R., Fegan, M., Hayward, A., Krishnapillai, V., and Hong, W. 1997. A novel method for development of species and strain-specific DNA probes and PCR primers for identifying *Burkholderia solanacearum* (formerly *Pseudomonas solanacearum*). *Asia Pac. J. Mol. Biol. Biotechnol.* 5:19-30.
- Ozakman, M., and Schaad, N. 2003. A real-time BIO-PCR assay for detection of *Ralstonia solanacearum* race 3, biovar 2, in asymptomatic potato tubers. *Can. J. Plant Pathol.* 25:232-239.
- Pastrik, K. H., and Maiss, E. 2000. Detection of *Ralstonia solanacearum* in potato tubers by polymerase chain reaction. *J. Phytopathol.* 148:619-626.
- Poussier, S., Chéron, J.-J., Couteau, A., and Luisetti, J. 2002. Evaluation of procedures for reliable PCR detection of *Ralstonia solanacearum* in common natural substrates. *J. Microbiol. Methods* 51:349-359.
- Poussier, S., Prior, P., Luisetti, J., Hayward, C., and Fegan, M. 2000. Partial sequencing of the *hrpB* and endoglucanase genes confirms and expands the known diversity within the *Ralstonia solanacearum* species complex. *Syst. Appl. Microbiol.* 23:479-486.
- Pradhanang, P., Elphinstone, J., and Fox, R. 2000. Sensitive detection of *Ralstonia solanacearum* in soil: A comparison of different detection techniques. *Plant Pathol.* 49:414-422.
- Prior, P., and Fegan, M. 2005. Diversity and molecular detection of *Ralstonia solanacearum* race 2 strains by multiplex PCR. Pages 405-414 in: *Bacterial Wilt Disease and the Ralstonia solanacearum Species Complex*. C. Allen, P. Prior, and A. Hayward, eds. American Phytopathological Society, St. Paul, MN.
- Rajendram, D., Ayenza, R., Holder, F. M., Moran, B., Long, T., and Shah, H. N. 2006. Long-term storage and safe retrieval of DNA from microorganisms for molecular analysis using FTA matrix cards. *J. Microbiol. Methods* 67:582-592.
- Remenant, B., Babujee, L., Lajus, A., Médigue, C., Prior, P., and Allen, C. 2012. Sequencing of K60, type strain of the major plant pathogen *Ralstonia solanacearum*. *J. Bacteriol.* 194:2742-2743.
- Scherf, J. M., Milling, A., and Allen, C. 2010. Moderate temperature fluctuations rapidly reduce the viability of *Ralstonia solanacearum* race 3, biovar 2, in infected geranium, tomato, and potato plants. *Appl. Environ. Microbiol.* 76:7061-7067.
- Schönfeld, J., Heuer, H., van Elsland, J. D., and Smalla, K. 2003. Specific and sensitive detection of *Ralstonia solanacearum* in soil on the basis of PCR amplification of *fliC* fragments. *Appl. Environ. Microbiol.* 69:7248-7256.

47. Seal, S. E., Jackson, L. A., and Daniels, M. J. 1992. Isolation of a *Pseudomonas solanacearum*-specific DNA probe by subtraction hybridization and construction of species-specific oligonucleotide primers for sensitive detection by the polymerase chain reaction. *Appl. Environ. Microbiol.* 58: 3751-3758.
48. Stefani, E., Giosue, S., and Mazzucchi, U. 2005. Detection of latent infections of *Ralstonia solanacearum* biovar 2, race 3 in tomato crops. *J. Plant Pathol.* 87: 167-171.
49. Strider, D., Jones, R., and Haygood, R. 1981. Southern bacterial wilt of geranium caused by *Pseudomonas solanacearum*. *Plant Dis.* 65:52-53.
50. Stulberg, M., Shao, J., and Huang, Q. 2015. A multiplex PCR assay to detect and differentiate select agent strains of *Ralstonia solanacearum*. *Plant Dis.* 99: 333-341.
51. Swanson, J. K., Montes, L., Mejia, L., and Allen, C. 2007. Detection of latent infections of *Ralstonia solanacearum* race 3 biovar 2 in Geranium. *Plant Dis.* 91:828-834.
52. Swanson, J. K., Yao, J., Tans-Kersten, J., and Allen, C. 2005. Behavior of *Ralstonia solanacearum* race 3 biovar 2 during latent and active infection of geranium. *Phytopathology* 95:136-143.
53. Tans-Kersten, J., Guan, Y., and Allen, C. 1998. *Ralstonia solanacearum* pectin methylesterase is required for growth on methylated pectin but not for bacterial wilt virulence. *Appl. Environ. Microbiol.* 64:4918-4923.
54. Temple, T. N., and Johnson, K. B. 2011. Evaluation of Loop-Mediated Isothermal Amplification for rapid detection of *Erwinia amylovora* on pear and apple fruit flowers. *Plant Dis.* 95:423-430.
55. USDA-APHIS. 2005. Part II. 7 CFR Part 331 and 9 CFR Part 121 Agricultural Bioterrorism Protection Act of 2002; Possession, use, and transfer of biological agents and toxins; final rule. *Fed. Regist.* 70:13241-13292.
56. van Elsas, J., Van Overbeek, L., and Trigalet, A. 2005. The viable but non-culturable state in *Ralstonia solanacearum*: Is there a realistic threat to our strategic concepts? Pages 103-119 in: *Bacterial Wilt Disease and the Ralstonia solanacearum Species Complex*. C. Allen, P. Prior, and A. Hayward, eds. American Phytopathological Society, St. Paul, MN.
57. van Elsas, J. D., Kastelein, P., de Vries, P. M., and van Overbeek, L. S. 2001. Effects of ecological factors on the survival and physiology of *Ralstonia solanacearum* bv. 2 in irrigation water. *Can. J. Microbiol.* 47:842-854.
58. Weller, S. A., Elphinstone, J. G., Smith, N. C., Boonham, N., and Stead, D. E. 2000. Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. *Appl. Environ. Microbiol.* 66:2853-2858.
59. Williamson, L., Nakaho, K., Hudelson, B., and Allen, C. 2002. *Ralstonia solanacearum* race 3, biovar 2 strains isolated from geranium are pathogenic on potato. *Plant Dis.* 86:987-991.
60. Wilson, I. G. 1997. Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.* 63:3741-3751.
61. Wimer, A. F., Rideout, S. L., and Freeman, J. H. 2011. Temporal and spatial distribution of tomato bacterial wilt on Virginia's Eastern Shore. *HortTechnology* 21:198-201.
62. Xin, Z., Velten, J. P., Oliver, M. J., and Burke, J. J. 2003. High-throughput DNA extraction method suitable for PCR. *Biotechniques* 34:820-827.