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# Recombinase Polymerase Amplification Assay for Rapid Field Diagnosis of Stewart's Wilt of Corn Pathogen *Pantoea stewartii* subsp. *stewartii*

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**Abstract:** Stewart's vascular wilt and leaf blight of sweet corn is caused by the Gram-negative enteric bacterium *Pantoea stewartii* subsp. *stewartii*. Stewart's wilt results in substantial yield losses worldwide warranting rapid and accurate disease diagnosis. Recombinase polymerase amplification (RPA) is an isothermal technique that is tolerant to host plant-derived inhibitors and is, therefore, ideally suited for rapid in-field detection *vis-à-vis* traditional polymerase chain reaction-based molecular assays. An RPA assay coupled with a Lateral Flow Device (LFD) was developed for rapid, accurate, and sensitive real-time detection of *P. stewartii* subsp. *stewartii* directly from the infected host offering in-field pathogen detection, timely disease management, and satisfying quarantine and phytosanitary requirements. Twelve novel primer sets were designed against conserved genomic regions of *P. stewartii* subsp. *Stewartii*; however, only the primers for amplification of the intergenic spacer region between capsular polysaccharide genes *cpsA* and *cpsB* were discernibly unique and adequate for unambiguous identification of *P. stewartii* subsp. *stewartii*. The *P. stewartii* subsp. *stewartii*-specific primers were further validated in a simplex RPA assay for specificity against twenty-six bacterial species representing several *Pantoea* and other closely related bacterial species/subspecies/strains found in the same niche, and naturally or artificially infected plant samples. The integrated RPA/LFD assay was also optimized for rapid and sensitive on-site detection of *P. stewartii* subsp. *stewartii* with an empirical detection limit of 0.0005 pg $\mu L^{-1}$ bacterial DNA and $1 \times 10^2$ CFU $mL^{-1}$ (app. two bacterial cells used per RPA reaction) in minimally processed samples for accurate, low-cost, and point-of-need diagnosis of the quarantine pathogen *P. stewartii* subsp. *stewartii*.

**Keywords:** corn leaf blight; Lateral Flow Device; *Pantoea stewartii* subsp. *stewartii*; phytosanitation; recombinase polymerase amplification; Stewart's wilt



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## 1. Introduction

*Pantoea* genus (γ-proteobacteria; *Enterobacteriaceae*) is present across diverse ecological niches and hosts. *Pantoea stewartii* subsp. *stewartii* [previously *Erwinia stewartii* (Smith) Dye] is an aerobic Gram-negative enteric bacterium that primarily infects field and sweet corn (*Zea mays*) causing Stewart's wilt and leaf blight. Infection at the young seedling stage is marked by water-soaked lesions of young expanding leaves, progressing to severe wilting and eventual death of the young seedlings [1,2]. The mature plants, if infected after the emergence of the tassels, are prone to leaf blight due to bacterial colonization of the xylem. Leaf blight presents symptoms such as long linear yellow-gray lesions with a wavy margin running parallel to the leaf veins. These lesions later turn necrotic and dark in color [1]. *P. stewartii* subsp. *stewartii* is almost exclusively vectored by the corn flea beetle *Chaetocnema*

*pulicaria* Melsheimer (Coleoptera; *Chrysomelidae*). Early maturing sweet corn varieties and some elite inbred maize lines are susceptible to Stewart's wilt disease. Despite availability of resistant corn varieties, resistance may not always prove adequate due to severity of flea beetle infestations, growth stage of infected seedlings, restricted planting window for processing and fresh market corn [2]. Yield losses frequently range from 40 to 100% when susceptible sweet corn hybrids grown under epidemic conditions are infected prior to the five-leaf stage [3].

*P. stewartii* subsp. *stewartii* has been reported as the causal agent of bacterial leaf wilt disease in sugarcane (*Saccharum officinarum*) [4]. Lately, jackfruit (*Artocarpus heterophyllus*) bronzing disease—due to *P. stewartii* subsp. *stewartii* infection—is causing significant economic losses in Malaysia [5]. Choi et al. [6] reported an outbreak of the occurrence of Stewart's wilt on Chinese bamboo (*Dracaena sanderiana*) using physiological and molecular analyses and pathogenicity tests. The host range of *P. stewartii* subsp. *stewartii* likely extends to several other *Poaceae* species such as rice (*Oryza sativa*), oat (*Avena sativa*), and common wheat (*Triticum aestivum*), as well as palm (*Bactris gasipaes*) [7]. As a consequence, quarantine restrictions have been imposed by many countries due to seed transmission of Stewart's wilt [8], out of concern for emerging diseases [4–6], and due to asymptomatic infections in other crops [7]. Phytosanitation regulations can severely impact international trading as well as increase the surveillance and diagnostic costs at the point of origin. Some *Pantoea* species also pose a significant threat of nosocomial infections. Notably, *P. agglomerans* (previously designated *Erwinia herbicola* or *Enterobacter agglomerans*) has been implicated in several outbreaks of septicemia [9].

*Pantoea* are commonly associated with plant hosts as epiphytes or pathogens [10], thereby underscoring a critical need for molecular and phylogenetic characterization at the species and infraspecies levels. *Pantoea* genus was historically distinguished into seven species: *P. agglomerans*, the type species of the genus; *P. ananatis*; *P. stewartii* (divided into the two subspecies *stewartii* and *indologenes*); *P. dispersa*; *P. citrea*; *P. punctata;* and *P. terrea*; however, recent advances in molecular taxonomy has resulted in the identification of several new *Pantoea* species, and currently *Pantoea* genus comprises 29 valid and distinct published species [11]. *Pantoea* species/subspecies and strains are often marked by heterogeneous biochemical or nutritional characteristics and breach of species boundaries due to homologous recombination and lateral gene transfer events [12]. Consequently, understanding the phylogeny and interrelationships within the *Pantoea* genus has proven difficult relying solely on the 16S rRNA gene and other single-gene or limited multi-gene approaches.

Unknown plant isolates are usually assigned to the genus *Pantoea* following phenotypic tests such as colony color and Gram-staining but stringent molecular methods are needed for unambiguous taxonomic identification. Brady et al. [13,14] used fluorescent amplified fragment length polymorphism (FAFLP) and multilocus sequence analysis (MLSA) for molecular identification of the genus Pantoea at the species level, and suggested the existence of ten potential novel *Pantoea* species and the possible inclusion of *Pectobacterium cypripedii* in the genus Pantoea. Several methods, such as the enzyme-linked immunosorbent assay (ELISA) [15], PCR primers specific for virulence genes [8], TaqMan® primers for the *cpsD* gene (syn. *wceL*, for biosynthesis of the exopolysaccharide (EPS) stewartan) [16], genomic fingerprinting using 10-mer 'miniprimers' [17], and the 16S rRNA gene PCR-coupled ligase chain reaction (LCR) assay utilizing isotopic primers [18] have been used for species-level identification of *P. stewartii*. Molecular distinction of *P. stewartii* from other related species was achieved using matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI–TOF MS) analysis and specific diagnosis using both conventional and quantitative PCR [19]. Nonetheless, these methods were inadequate for differentiation between *P. stewartii* subsp. *stewartii* and subsp. *indologenes*. *P. stewartii* subsp. *indologenes* [20] is nonpathogenic on maize but causes leaf spots on foxtail millet (*Setaria italica*) and pearl millet (*Pennisetum americanum*), rot of pineapple (*Ananas comosus*), and center rot of onion (*Allium cepa*) [21]. Gehring et al. [22] used a stepdown PCR method to distinguish between the two *P. stewartii* subsp. based on the conserved single-nucleotide

polymorphisms (SNPs) in the housekeeping genes encoding recombinase A (*recA*) and UDP-glucose 4-epimerase (*galE*) and subtyping using MALDI–TOF MS protein fingerprints.

Precise taxonomic identification of the two *P. stewartii* subsp. and related strains remains time-consuming to date. Diagnostic assays include plant pathogenicity assays, gram staining (KOH test), the motility test, the Hugh-Leifson oxidation/fermentation test, the indole test with Kovacs reagent, esculin hydrolysis, and sequencing analyses. Furthermore, large-scale confirmatory PCR assays are cost-prohibitive due to instrumentation and require the use of nucleic acid extraction procedures marred by coextraction of host plant-derived inhibitors. The accuracy of PCR data from field samples may also be compromised if the host has mixed infections or if the target organism is present in low titers leading to false negative results.

Recombinase polymerase amplification (RPA) is an isothermal DNA amplification and detection technology [23]. Lately, RPA has positioned itself as a promising, cost-effective, highly sensitive and selective isothermal amplification technique capable of rapidly amplifying as low as 1–10 DNA target copies from minimally processed samples at constant (near)ambient temperatures (37–42 °C). In contrast to other routinely used molecular diagnostic tools, RPA remains highly affordable and amenable to point-of-care testing of clinical samples and on-site field testing [24]. Current commercial RPA kits use the *Sau* DNA polymerase from *Staphylococcus aureus* having the strand-displacement activity necessary for primer extension. Creatine kinase catalyzes phosphocreatine to creatine (Lohmann reaction) to generate ATP for the RPA reaction. The *Escherichia coli* RecA recombinase binds single-stranded oligonucleotide primers and probes in the presence of ATP and a high-molecular-weight macromolecular crowding agent polyethylene glycol to initiate the RPA process. The nucleoprotein filament interrogates the double-stranded DNA (dsDNA) target and invades the dsDNA at the cognate site. Primer hybridization results in the formation of the D-loop where the locally separated complementary DNA strand is stabilized using single-strand DNA binding (SSB) proteins to prevent the ejection of the inserted primer by branch migration. Cyclic iterations of primer hybridization, recombinase disassembly, and strand-displacing *Sau* DNA polymerase activity substitute for the heat denaturation step of the conventional PCR, resulting in the exponential amplification reaction. Commercial RPA kit configurations (TwistDx) enable DNA or RNA amplification, and RPA end-point products can be detected using agarose gel electrophoresis [23], TwistAmp™ exo probes (TwistDx, Cambridge, UK) [25,26], or lateral flow dipstick assays (MileniaBiotec, Giessen, Germany) [27–30].

RPA-based molecular toolkits have been developed for several agronomically important phytopathogens including DNA [31,32] and RNA [30,33,34] viruses, nematodes [35], bacterial [26,36,37], and fungal [27,29,38,39] species. To date, field diagnosis of rice leaf blight pathogen *P. ananatis*—using an integrated RPA-lateral flow dipstick assay [40]—remains the only diagnostic tool for characterizing any *Pantoea* species in a non-laboratory setting. The objectives of the current research were two-fold: (a) design and validate PCR primers for precise molecular identification and differentiation of *P. stewartii* subsp. *stewartii* from subsp. *indologenes* and other related bacteria colonizing corn, and (b) develop a portable, rapid, and sensitive molecular toolkit for point-of-need testing of Stewart's wilt pathogen in the field and in quarantine clinics.

## 2. Materials and Methods

### *2.1. Isolation and Storage of Bacterial Strains*

In total, twenty-six bacterial strains were used in this study (Table 1), including five *P. stewartii* subsp. *Stewartii*, four *P. stewartii* subsp. *Indologenes*, and seventeen other bacteria from closely related genera found in the niche. Some of these bacterial strains were previously isolated from naturally infected plant material and were curated in our lab repository. A few strains were isolated from the plant material intercepted at the quarantine diagnostic laboratory at Guangzhou Customs (Guangdong Province, Guangzhou, Tianhe District, China). All bacterial strains were cataloged and cryopreserved following confirmation using 16S

sequencing. All the strains were grown at 28 °C in Nutrient Broth (NB) containing 1 g yeast extract, 3 g beef extract, 5 g polypeptone, and 10 g sucrose $L^{-1}$ medium. To obtain pure cultures, 10 µL of bacterial suspension was streaked on NB supplemented with 15 g $L^{-1}$ agar (NA) for isolation of single-cell colonies.

**Table 1.** Bacterial strains used in this study.

| Bacterial Species/Strain | Repository [a] | Type Strain GenBank Acc. No. (Genome Size) |
|---|---|---|
| *Pantoea stewartii* subsp. *stewartii* | | |
| LX-2-8.19 | Field isolate, Sanya, China | |
| ATCC 8199 | ATCC | |
| ATCC 29227 | ATCC | DC283 (GCA_000248395.2, 5 Mb) |
| ATCC 8200 | ATCC | |
| ATCC 29228 | ATCC | |
| *Pantoea stewartii* subsp. *indologenes* | | |
| ATCC 35396 | ATCC | LMG 2632 (GCA_000757405.2, 4.7 Mb) |
| 4270-6 | Guangzhou Customs, China | |
| *Pantoea stewartii* | | |
| 4270-4 | Guangzhou Customs, China | ZJ-FGZX1 (GCA_011044475.1, 5 Mb) |
| 1082-3 | Guangzhou Customs, China | |
| *Pantoea agglomerans* 1848-Lin | Guangzhou Customs, China | ZJU23 (GCA_021559955.1, 5.1 Mb) |
| *Pantoea ananatis* DSM 30070 | DSMZ | LMG 5342 (GCA_000283875.1, 4.9 Mb) |
| *Pantoea cypripedii* ICMP 1591 | ICMP | LMG 2657 (GCA_002095535.1, 6.6 Mb) |
| *Acidovorax avenae* subsp. *avenae* ATCC 19307 | ATCC | AA81_1 (GCA_003029685.1, 5.8 Mb) |
| *Burkholderia andropogonis* ATCC 23060 | ATCC | Ba3549 (GCA_000566705.1, 6.2 Mb) |
| *Burkholderia cepacia* LMG 1222 | LMG | BC16 (CA_009586235.1, 3.4 Mb) |
| *Burkholderia gladioli* NCPPB 1888 | NCPPB | BBB-01 (GCA_016698705.1, 8.2 Mb) |
| *Clavibacter michiganensis* subsp. *nebraskensis* NCPPB 2578 | NCPPB | 61-1 (GCA_009739635.2, 3.1 Mb) |
| *Dickeya chrysanthemi* ICMP 10850 | ICMP | Ech1591 (GCA_000023565.1, 4.8 Mb) |
| *Pectobacterium atrosepticum* NCPPB 549 | NCPPB | JG10-08 (GCA_000696465.1, 5.0 Mb) |
| *Pectobacterium carotovorum* subsp. *carotovorum* 2412-1 | Field isolate, Sanya, China | PCCS1 (GCA_015277635.1, 4.9 Mb) |
| *Pseudomonas fluorescens* LX-2 | Field isolate, Sanya, China | 2P24 (GCA_002865505.1, 6.6 Mb) |
| *Pseudomonas fuscovaginae* NCPPB 3734 | NCPPB | CB98818 (GCA_000280575.1, 6.5 Mb) |
| *Pseudomonas syringae* pv. *panici* 2357-1 | Field isolate, Sanya, China | LMG 2367 (GCA_000282735.1, 6 Mb) |
| *Pseudomonas syringae* pv. *syringae* LMG 5083 | LMG | B48 (GCA_030035225.1, 6.1 Mb) |
| *Xanthomonas albilineans* ICMP196 | ICMP | Xa-FJ1 (GCA_009931595.1, 3.8 Mb) |
| *Xanthomonas axonopodis* pv. *vasculorum* ATCC13901 | ATCC | NCPPB 796 (GCA_013177355.1, 4.9 Mb) |

[a] ATCC, American Type Culture Collection, Manassas, VA, USA; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; ICMP, International Collection of Microorganisms from Plants, Aukland, New Zealand; LMG, Collection Laboratorium voor Microbiologie Universiteit Gent, Gent, Belgium; NCPPB, National Collection of Plant Pathogenic Bacteria, York, UK.

### 2.2. Validation of Primers for the Detection of *P. stewartii subsp. stewartii*

Twelve novel pairs of primers were designed for the identification of *P. stewartii* subsp. *stewartii*. All the primer/probe sequences used for the regular genomic PCR and RPA assays are summarized in Table 2. Specificity of these primers and probes was evaluated in silico using the NCBI GenBank BLASTn tool [41]. Bacterial cultures were grown in NB at 28 °C and 200 rpm for 16 h and harvested at mid-log phase for extraction of genomic DNA. Naturally and artificially infected plants were scored for disease and symptomatic lesions were excised using sterile scalpel blades. Genomic DNA from pure bacterial cultures and infected plant material was extracted using the TIANamp Bacteria DNA Kit (Tiangen Biotech Co. Ltd., Beijing, China) following the manufacturer's protocol. Two microliters of DNA template (app. 100 ng) was used for PCR reaction containing 12.5 µL of 2× *Taq* Plus DNA Polymerase Master Mix (Tiangen), 1 µL of primers (up/down, 10 µM) and 9.5 µL of nuclease-free water. The thermocycling protocol was as follows: initial denaturation at 95 °C for 5 min; 35× cycles of denaturation at 95 °C for 30 s; primer annealing at 60 °C for 30 s; extension at 72 °C for 1 min; followed by final extension at 72 °C for 10 min. The

amplification products were resolved using agarose gel electrophoresis and their fidelity was verified using Sanger sequencing.

**Table 2.** Primers and probe sets used for the detection of *Pantoea stewartii* subsp. *stewartii*.

| Primer/Probe | Sequence (5′ → 3′) | Amplicon (bp) | Gene (GenBank Acc. No.) |
|---|---|---|---|
| AB inter F1<br>AB inter R1 | TGGATTTTATGCTGTGGTACTATGAAAACGGT<br>TTGAATAATAGGTAATCATTCTGTTTTGTCTGC | 150 | methyl-accepting chemotaxis protein (*mcp*, DSJ_RS03345) |
| AB inter F2<br>AB inter R2 | AAATGGATTTTATGCTGTGGTACTATGAAAAC<br>AATAATAGGTAATCATTCTGTTTTGTCTGCACT | 150 | |
| GalE F1<br>GalE R1 | GAATTCATTATCCGTGATTTTGCCAAAG<br>CTTTATAACCTTCAATTTTGTCCAGATGATC | 304 | UDP-glucose 4-epimerase (*gale*, DSJ_RS16255) |
| GalE F2<br>GalE R2 | GATGGTCGAATTCATTATCCGTGATTTTG<br>TATAACCTTCAATTTTGTCCAGATGATCCAG | 311 | |
| MDC283galE<br>DC283galEc | CGACCTGTTTGCCTCTCACT<br>CATCAGCTTGGAGGTGCCA | 268 | |
| DC283galE<br>DC283galEc | AATATTACGAAAATAACGTTGC<br>CATCAGCTTGGAGGTGCCA | 182 | |
| p-g-F<br>p-g-R | GGGATTCACGCGTTTCATTTATTTGATCTTGC<br>TCATGCAAATATCCTCAGTCAACTCGCCAAAA | 165 | glutamine:fructose-6-phosphate amino transferase (*gfat*, DSJ_RS02230) |
| Pss F1<br>Pss R | TATTGATCGTATCCTCATTGTTGCTT<br>GCGCTCTGGCTATATTGGGTTATTACGGCAC | 189 | |
| Pss F2<br>Pss R | GCTGCAGGTTATTGATCGTATCCTCATTG<br>GCGCTCTGGCTATATTGGGTTATTACGGCAC | 195 | |
| EGaseUP<br>EGaseNP | GGCGGCGGTGAAAGAGTT<br>GATGCACCGACGGAAACAA | 453 | DNA-directed RNA polymerase subunit beta (*rpoB*, DSJ_RS01980) |
| cpsAB2313F<br>cpsR | AGAAAACGCTGATGCCAGAC<br>ACTATCCTGACTCAGGCACT | 375 | Intergenic spacer region between stewartan (EPS) biosynthesis genes *cpsA* (DSJ_RS16295) and *cpsB* (DSJ_RS16315) |
| CLL001-F<br>CLL001-R | GGTAGAAAACGCTGATGCCAGACAGAACACCGTC<br>AAGTAAACTATCCTGACTCAGGCACTGAACATG | 256 | |
| CL001-PIexo | GTACCACAGCATAAAATCCATTTATTCAACAAATC<br>[FAM-dT][THF]CA[BHQ1-dT]<br>AAAAAAGCGGTACGGC [C3Spacer] | | |
| CL001-PII | FAM-<br>TACCACAGCATAAAATCCATTTATTCAACAAATC<br>[THF]CAAAAAAAGCGGTACGGC [C3Spacer] | | |

### *2.3. Inoculation of Maize Leaves and Seeds with P. stewartii subsp. stewartii*

*P. stewartii* subsp. *stewartii* strain ATCC 29277 was grown in NB at 28 °C and 200 rpm for 16 h. Bacteria in the exponential growth phase were harvested via centrifugation, washed two times, and re-suspended in sterile water to an $Abs._{600}$ = 0.3 (app. $1 \times 10^8$ CFU $mL^{-1}$). The third new leaves of 15-day-old corn seedlings were needle-inoculated and scored for lesion development [42]. Bacterial genomic DNA was extracted seven days post-inoculation (dpi) from the infected leaf material. Likewise, maize seeds were imbibed with bacterial suspension (200 µL per seed) for six hours. Seeds were allowed to dry at room temperature and stored at 4 °C until further use. Five infected seeds were rehydrated in 800 µL sterile water for four hours and macerated for DNA extraction for RPA analysis. Serial dilutions of *P. stewartii* subsp. *stewartii* cultures ($1 \times 10^8$ to $1 \times 10^2$ CFU $mL^{-1}$ were used for inoculation of leaf and seed tissue for validating RPA assay sensitivity for bacterial titer detection.

### *2.4. RPA Primer/Exo Probe Design and Assay*

RPA primers (Figure 1) were designed to be 30–35 nucleotides long without long runs of any one particular nucleotide or a large number of small repeats, 30–70% guanine–cytosine (GC) content, and yielding 80–400 bp amplicons [24]. Likewise, the exo probe (Figure 1) was designed manually to have the following characteristics when possible: 30–70% guanine–cytosine (GC) content, avoiding runs of more than four identical nucleotides and secondary structures with a melting temperature (Tm) between 37 and 42 °C. The exo probe is a long oligonucleotide (46–52 bases) with 30 and 15 nucleotides placed at 5′ and 3′, respectively, to an internal base analog tetrahydrofuran (THF). THF is located between a fluorophore 5(6)-carboxyfluorescein (FAM) and a quencher (e.g., Black Hole Quencher 1 or Black Hole Quencher 2) with a blocked 3′ end (such as 3′ phosphate group or dideoxy nucleotide). All the primers and probes were purchased from Beijing Genomics Institute (BGI Group, Shenzhen, China).

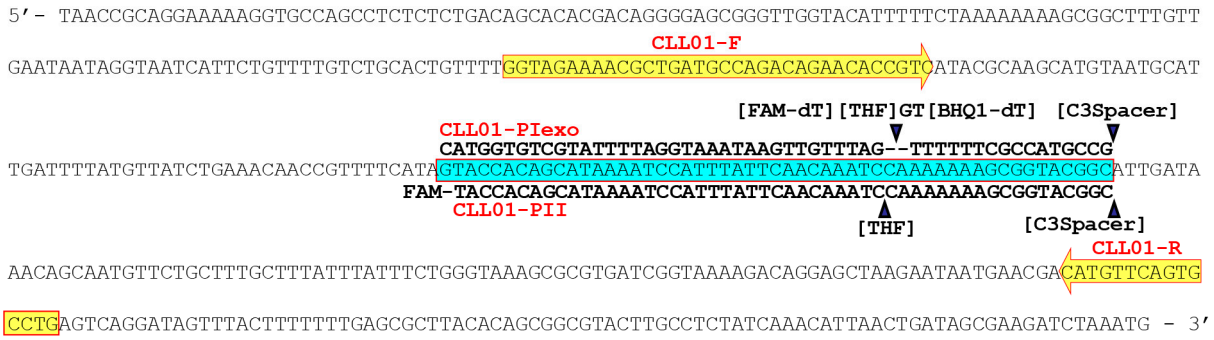


**Figure 1.** Simplex RPA assay designed to specifically detect *Pantoea stewartii* subsp. *stewartii*. The primers CLL011-F/R and the exo probe CLL001-PIexo amplify the 256 bp intergenic spacer region between stewartan (EPS) biosynthesis genes *cpsA* (DSJ_RS16295) and *cpsB* (DSJ_RS16315) in RPA reaction. Probe CLL001-PII is used for detection of amplified products in an integrated RPA/LFD assay. THF, tetrahydrofuran residue; FAM, 5(6)-carboxyfluorescein; BHQ-1, Black Hole Quencher 1.

RPA reactions were performed in 50 µL volume using the DNA Isothermal Rapid Amplification Kit from Amplification Future (Changzhou) Biotechnology Co., Ltd., Changzhou, China. Two microliters of RPA primers (forward and reverse; 10 µM), 0.6 µL of exo probe (10 µM), and 11.4 µL of nuclease-free $H_2O$ were mixed with 29.4 µL of rehydration buffer A to make the master mix. The master mix was distributed into 0.2 mL reaction tubes containing the lyophilized RPA enzyme powder. Next, 2 µL of template DNA was pipetted into the reaction tubes. The internal and blank control templates were treated in parallel. Finally, 2.5 µL of 280 mM magnesium acetate (Buffer B) was added to each tube. The reactions were gently mixed and centrifuged briefly. The tubes were immediately placed in a portable isothermal nucleic acid detection system (Isothermal Fluorescence Detector WL-16-III; Amplification Future, Changzhou, China). The fluorescence signal was collected via the FAM channel every 30 s for 20 min (end-point reading) at 37 °C in real-time which increased markedly with successful amplification. For the RPA assay, using artificially infected leaf or seed material, 13.4 µL of DNA extract was used in the reaction mix.

### *2.5. RPA/Lateral Flow Dipstick (LFD) Assay for On-Site Detection of P. stewartii subsp. stewartii*

LFD assay probes were marked by FAM at the 5′ end and THF as the *E. coli* endonuclease IV (nfo) target in the middle with a blocked 3′ end. The RPA reaction components were mixed as detailed above and incubated at 39 °C in a water bath for 10 min. Alternatively, in the field, app. four $cm^2$ leaf tissues with lesions were coarsely macerated by hand in a plastic bag containing 1 mL of sterile water. Ten microliters of crude extract was mixed with the RPA reaction and the tubes were incubated in closed palm at body temperature. A total of 50 µL of RPA product was applied to the receptacle of a portable lateral flow device (Amplification Future, Changzhou, China) containing two different detection lines coupled

with one positive control line to enable specific detection of FAM-labeled amplicons visible to the unaided eye within 10 min.

## 3. Results

### *3.1. Validation of Unique Primer Sets for Detection of P. stewartii subsp. stewartii*

Twelve novel and unique primer sets for specific detection of the genus *P. stewartii* subsp. *stewartii* strain DC283 (reference strain, GeneBank acc. no. GCA_000248395.2) were designed (Table 2) for partial amplification of genes encoding the methyl-accepting chemotaxis protein (DSJ_RS03345), UDP-glucose 4-epimerase (UGE; EC:5.1.3.2) (*gale*; DSJ_RS16255), glutamine:fructose-6-phosphate aminotransferase (GFAT; EC:2.6.1.16) (DSJ_RS02230), DNA-directed RNA polymerase subunit beta (*rpoB*; DSJ_RS01980), and the intergenic spacer region between capsular polysaccharide genes *cpsA* (DSJ_RS16295) and *cpsB* (DSJ_RS16315). These genomic regions were identified through comparative genomic analyses and in silico validation for query coverage and % identity. Initial specificity tests were performed with endpoint PCR (Figure 2, Table 3). However, only the primer pair CLL001F/R targeted against the *cpsA/cpsB* intergenic spacer region yielded specific and unambiguous amplification products for all the tested *P. stewartii* subsp. *stewartii* strains (LX-2-8.19, ATCC 8199, ATCC 8200, ATCC 29227, ATCC 29228, and DC283) without any false positive or negative amplification data (Figure 2A). The end-point PCR assay was evaluated using twenty-six bacterial strains, representing five *P. stewartii* subsp. *stewartii*, four *P. stewartii* subsp. *indologenes* and seventeen other related bacteria isolated from the same niche. Similar amplification results were obtained for pure bacterial cultures and naturally or artificially infected host tissues. The remaining 11 primer sets amplified genomic DNA from other related bacterial strains precluding their utility for specific identification of *P. stewartii* subsp. *stewartii.* For example, the primer set MDC283galE/DC283galEc showed amplification for all the tested strains of *P. stewartii* subsp. *stewartii* as well as for *P. stewartii* subsp. *indologenes* strains ATCC 35396 and 4270-6, *P. stewartii* strains 4270-4 and 1082-3, *P. cypripedii* strain ICMP 1591, and *P. ananatis* strain DSM 30070 (Figure 2B).

### *3.2. Optimization of Simplex RPA Assay for Detection of P. stewartii subsp. stewartii*

PCR-validated specific primers and an exo probe (CL001-$PI_{exo}$) (Table 2) designed against the *cpsA/cpsB* intergenic spacer region were further evaluated for optimization of a simplex RPA assay for the detection of *P. stewartii* subsp. *stewartii*. To this end, genomic DNA isolated from the cultured *P. stewartii* subsp. *stewartii* strain DC283 was used as the positive control and sterile water as the negative control. To determine the optimal reaction temperature of the RPA assay, the reactions were performed at various temperatures between 37 and 42 °C for 20 min, and the amplicons were monitored using a portable fluorescence detector. The optimum RPA reaction temperature was found to be 37 °C. Higher incubation temperatures had no discernible effect on the amplification reaction. The DNA extracted from sequence-verified *P. stewartii* subsp. *stewartii* strain LX-2-8.19 routinely gave positive amplification curves [threshold time, TT(F) =13–17 min]. In a representative experiment, the TT(F) values for *P. stewartii* subsp. *stewartii* strains ATCC 8199, ATCC 8200, ATCC 29227, and ATCC 29228 were found to be 16.0, 10.8, 13.4, and 10.5 min, respectively. Under similar assay conditions, the *P. stewartii* subsp. *stewartii* RPA assay—the exo probe and primer set—amplified only the DNA extracted from the five strains of subsp. *stewartii* but not that from four *P. stewartii* subsp. *indologenes* strains or any other related bacterial species tested (Figure 3A–C).

The sensitivity of the RPA assay was evaluated using serially diluted template DNA of strain ATCC 29277 (5, 0.5, and 0.05 ng $\mu L^{-1}$; 0.05, 0.005, and 0.0005 pg $\mu L^{-1}$). Adequate amplification was observed at 37 °C within 20 min in a reaction containing as low as 0.0005 pg $\mu L^{-1}$ target DNA [TT(F) value = 25.8 min] (Figure 3D). Symptomatic corn leaves from various locations in the field at Sanya (Hainan Province, South China) were collected for genomic DNA extraction and verified using end-point PCR amplification and sequencing. RPA amplification for *P. stewartii* subsp. *stewartii* strain LX-2 DNA was

observed in a field population of naturally infected maize seedlings. The RPA assay was also able to detect *P. stewartii* subsp. *stewartii* ATCC 29277 in the leaves of artificially infected maize seedlings [TT(F) value = 19–30 min] (Figure 3E) as well as in the mature and desiccated maize kernels [TT(F) value = 18–26 min]. The detection sensitivity of the RPA assay in desiccated seeds was determined to be $1 \times 10^2$ CFU $mL^{-1}$ (roughly equivalent to two bacterial cells) [TT(F) value = 26.1 min] (Figure 3F). Mock (sterile water)-inoculated leaves and seeds were used as negative controls in these experiments.

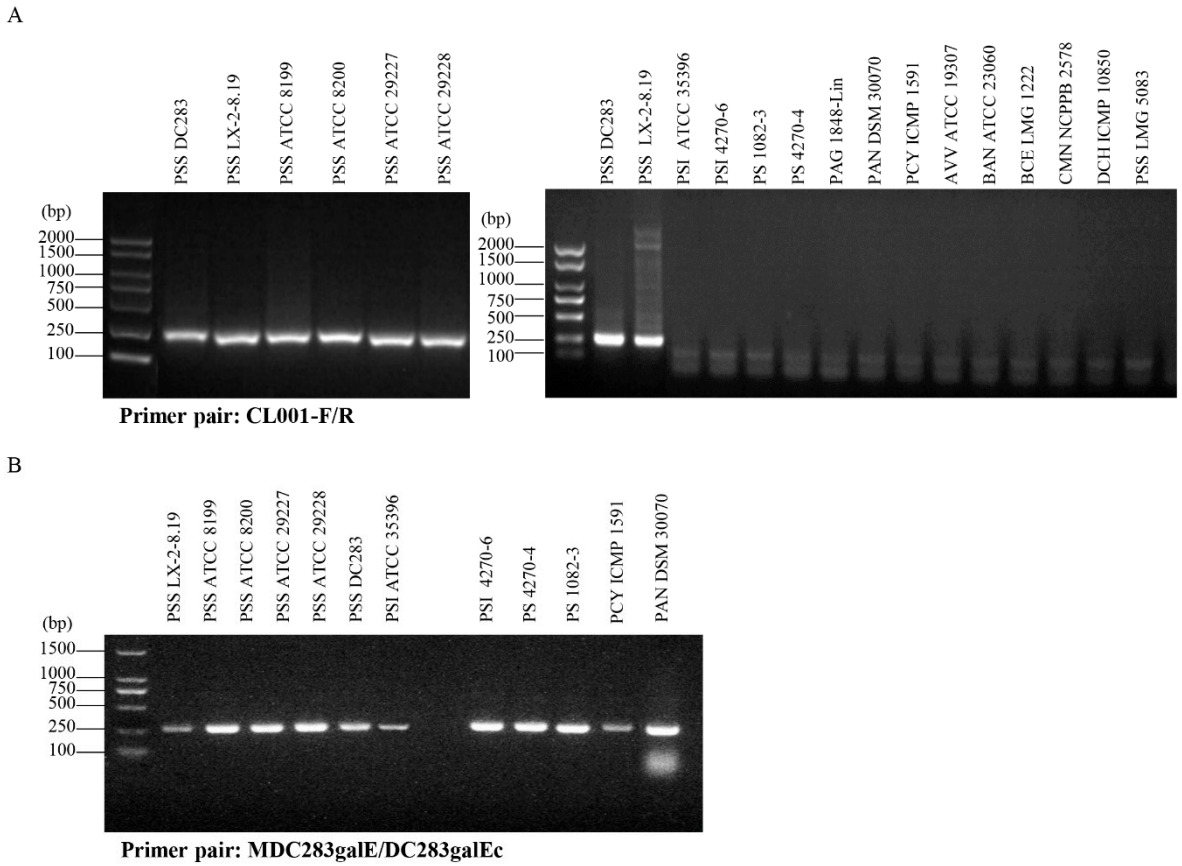


**Figure 2.** Validation of primer specificity in an end-point PCR assay for the detection of *Pantoea stewartii* subsp. *stewartii*: (**A**,**B**) Representative gels showing PCR amplification products using primer pairs CLL01-F/R (256 bp) and MDC283galE/DC283galEc (268 bp). The bacterial strains used are *P. stewartii* subsp. *stewartii* (PSS) strains DC283, LX-2-8.19, ATCC 8199, ATCC 8200, ATCC 29227, and ATCC 29228, *P. stewartii* subsp. *indologenes* (PSI) strains ATCC 35396 and 4270-6, *P. stewartii* (PS) strain 4270-4 and 1082-3, *P. agglomerans* (PAG) strain 1848-Lin, *P. ananatis* (PAN) strain DSM 30070, *P. cypripedii* (PCY) strain ICMP 1591, *Acidovorax avenae* subsp. *avenae* (AVV) strain ATCC 19307, *Burkholderia andropogonis* (BAN) strain ATCC 23,060, *B. cepacia* (BCE) strain LMG 1222, *Clavibacter michiganensis* subsp. *nebraskensis* (CMN) strain NCPPB 2578, *Dickeya chrysanthemi* (DCH) strain ICMP 10850, *Pseudomonas syringae* pv. *syringae* (PSP) strain LMG 5083. Note that the primer pair CL001-F/R amplifies the intergenic spacer region between *cpsA* and *cpsB* specifically for all the tested strains of *P. stewartii* subsp. *stewartii*.

### *3.3. RPA/LFD Assay for Point-of-Need Detection of P. stewartii subsp. stewartii*

Detection sensitivity of the *P. stewartii* subsp. *Stewartii*-specific RPA primers and probe (CL001-PII) (Table 2) were optimized using serially diluted genomic DNA of strain ATCC 29277. A detection limit of 0.0005 pg $\mu L^{-1}$ target DNA [TT(F) value = 25.8 min, Figure 3D] was readily discernible using the integrated RPA/LFD assay (Figure 4A). The RPA/LFD assay was further optimized using the DNA extracted from artificially inoculated maize seedlings. The DNA that was extracted from artificially inoculated maize leaf lesions scored

positive using the RPA/LFD assay for detection of *P. stewartii* subsp. *stewartii* strain ATCC 29277. Maize seeds were also artificially inoculated with a serially diluted pure culture of *P. stewartii* subsp. *stewartii* strain ATCC 29277 ($1 \times 10^8$ to $1 \times 10^2$ CFU $mL^{-1}$) and used for DNA extraction. Similar to the results obtained in the portable DNA fluorescence detection device, the RPA/LFD assay was able to detect the bacterial DNA at an inoculum density of $1 \times 10^2$ CFU $mL^{-1}$ (Figure 4B).

**Table 3.** Amplification specificity of primers designed for the detection of *Pantoea stewartii* subsp. *stewartii*.

| Bacterial Strains | Primer Pairs | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AB Inter F1/R1 | AB Inter F2/R2 | GalE F1/R1 | GalE F2/R2 | MDC283galE /DC283galEc | DC283galE /DC283galEc | p-g-F/R | Pss F1/R | Pss F2/R | EGaseUP/NP | cpsAB2313F /cpsR | CL001-F/R |
| *Pantoea stewartii* subsp. *stewartii* LX-2-8.19 | | | | | | | | | | | | |
| *P. stewartii* subsp. *stewartii* ATCC 8199 | | | | | | | | | | | | |
| *P. stewartii* subsp. *stewartii* ATCC 8200 | | | | | | | | | | | | |
| *P. stewartii* subsp. *stewartii* ATCC 29227 | | | | | | | | | | | | |
| *P. stewartii* subsp. *stewartii* ATCC 29228 | | | | | | | | | | | | |
| *P. stewartii* subsp. *indologenes* ATCC 35396 | | | | | | | | | | | | |
| *P. stewartii* subsp. *indologenes* 4270-6 | | ■ | | | | | | | | | | |
| *P. stewartii* 4270-4 | | | | | | | | | | | | |
| *P. stewartii* 1082-2 | | | | | | | | | | | | |
| *P. agglomerans* 1848-Lin | | | | | | | | | | | | |
| *P. ananatis* DSM 30070 | | | | | | | | | | | | |
| *P. cypripedii* ICMP 1591 | | | | | | | | | | | ■ | |
| *Acidovorax avenae* subsp. *avenae* ATCC 19307 | | | | | | | | | | | | |
| *Burkholderia andropogonis* ATCC 23060 | | | | | | | | | | | | |
| *Burkholderia cepacia* LMG 122 | | | | | | | | | | | | |
| *Burkholderia gladioli* NCPPB1888 | | | | | | | | | | | ■ | |
| *Clavibacter michiganensis* subsp. *nebraskensis* NCPPB2578 | | | | | | | ■ | | | | | |
| *Dickeya chrysanthemi* ICMP10850 | | | | | | | | | | | | |
| *Pectobacterium atrosepticum* NCPPB 549 | | | | | | | | | | | | |
| *Pectobacterium carotovorum* subsp. *carotovorum* 2412-1 | | | | | | | | | | | | |
| *Pseudomonas fluorescens* LX-2 | | | | | | | | | | | | |
| *Pseudomonas fuscovaginae* NCPPB 3734 | | | | | | | | | | | | |
| *Pseudomonas syringae* pv. *panici* 2357-1 | | | | | | | | | | | | |
| *Pseudomonas syringae* pv. *syringae* LMG 5083 | | | | | | | | | | | | |
| *Xanthomonas albilineans* ICMP196 | | | | | | | | | | | | |
| *Xanthomonas axonopodis* pv. *vasculorum* ATCC 13901 | | | | | | | | | | | | |

Gray box, expected amplification product; White box, no amplification; Black box, ambiguous results.

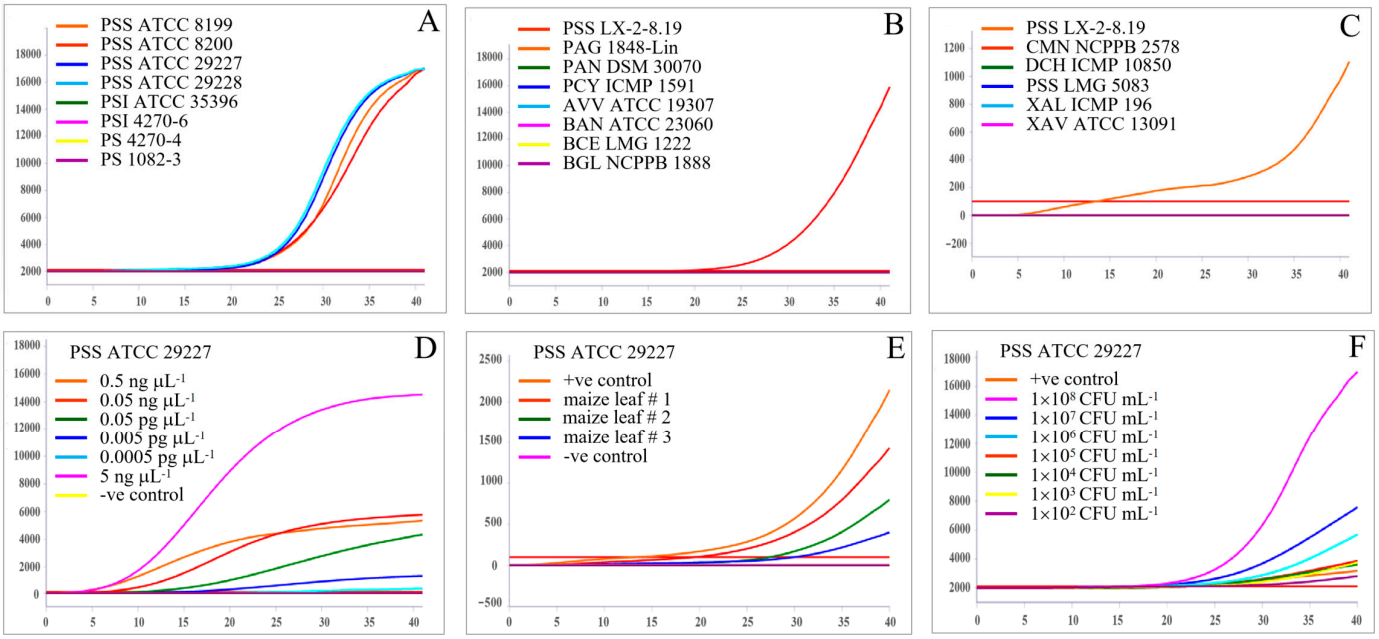


**Figure 3.** Simplex RPA assay for the detection of *Pantoea stewartii* subsp. *stewartii.* Amplification curves for RPA reactions incubated at 37 °C displayed using a portable isothermal nucleic acid detection system. Primer pair (CL001-F/R) and exo probe (CL001-PIexo) for amplification of *cpsA* and *cpsB* intergenic spacer region were used: (**A**) Amplification curves were observed for *P. stewartii* subsp. *stewartii* (PSS) strains ATCC 8199, ATCC 8200, ATCC 29227, and ATCC 29228. DNA extracted from *P. stewartii* subsp. *indologenes* (PSI) strains ATCC 35396 and 4270-6, and *P. stewartii* (PS) strains 4270-4 and 1082-3 did not yield any amplification; (**B**,**C**) DNA extracted from *P. agglomerans* (PAG) strain 1848-Lin, *P. ananatis* (PAN) strain DSM 30070, *P. cypripedii* (PCY) strain ICMP 1591, *Acidovorax avenae* subsp. *avenae* (AVV) strain ATCC 19307, *Burkholderia andropogonis* (BAN) strain ATCC 23060, *B. cepacia* (BCE) strain LMG 1222, *B. gladioli* (BGL) strain NCPPB 1888, *Clavibacter michiganensis* subsp. *nebraskensis* (CMN) strain NCPPB 2578, *Dickeya chrysanthemi* (DCH) strain ICMP 10850, *Pseudomonas syringae* pv. *syringae* (PSP) strain LMG 5083, *Xanthomonas albilineans* (XAL) strain ICMP 196, *X. axonopodis* pv. *vasculorum* (XAV) strain ATCC 13091 did not yield amplification curves. PSS strain LX-2-8.19 represents the positive control; (**D**–**F**) DNA amplification curves for PSS strain ATCC 29227 using serially diluted genomic DNA (**D**), artificially inoculated maize seedlings (**E**), and desiccated seeds (**F**), respectively. Genomic DNA extracted from pure culture of PSS strain ATCC 29227 was used as the positive control, and sterile water as the negative control. Maize seeds were imbibed in serially diluted bacterial cultures and their crude extracts were assayed for bacterial infection.

The robustness of the RPA/LFD assay was evaluated for minimal sample processing and in-field applicability. The reactions were carried out using crude sap from the infected plants and incubated in the palm of a closed hand without employing additional equipment. DNA extracts from symptomatic corn leaves collected from the field that were previously confirmed positive for *P. stewartii* subsp. *stewartii* infection also tested positive in the RPA/LFD assay (Figure 4C). Likewise, the RPA probe/primer assay also amplified DNA in the crude plant extracts on the LFD device (Figure 4C). Neither positive results nor cross-amplification were observed when mock-inoculated tissues were tested. These results confirmed that the RPA/LFD assay can specifically detect the *P. stewartii* subsp. *stewartii* directly from infected plant material without DNA purification or any specialized equipment.

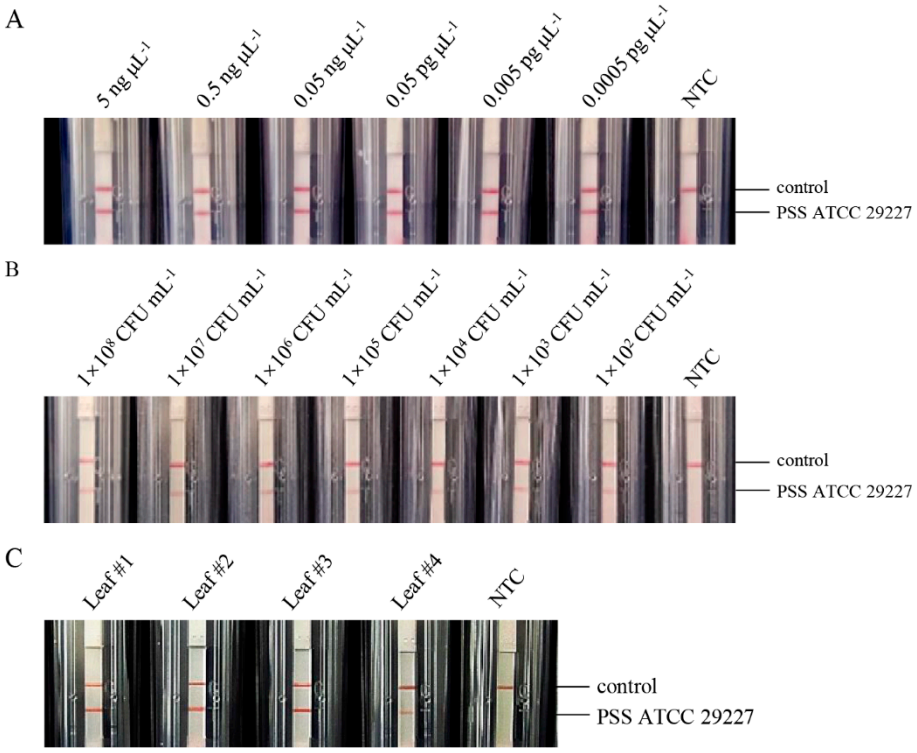


**Figure 4.** Simplex RPA/LFD assay for the detection of *Pantoea stewartii* subsp. *stewartii.* Primer pair (CL001-F/R) and probe (CL001-PII) were used for the amplification of *cpsA* and *cpsB* intergenic spacer region. LFD Nucleic Acid Detector allows the detection of FAM-labeled *P. stewartii* subsp. *stewartii* amplicons: (**A**) Serially diluted genomic DNA of *P. stewartii* subsp. *stewartii* strain ATCC 29227 was used as template for the RPA reaction; (**B**) RPA template DNA was extracted from maize seeds that were artificially infected using a serially diluted pure culture of *P. stewartii* subsp. *stewartii* strain ATCC 29227; (**C**) In-field RPA/LFD assay showing DNA amplification from template DNA extracted from field samples (leaf #1 and #2) or from crude leaf extracts (leaf #3 and #4). The RPA reactions were incubated at 37 °C in a water bath (**A**,**B**) and in a closed hand at body temperature (**C**). NTC, no template control.

## 4. Discussion

First introduced in 2006 [23], RPA has lately become the molecular diagnostic tool of choice for rapid (results within 5–20 min), simple (requiring minimally processed test material, few and easy hands-on steps), specific (single-target copy number and single-nucleotide polymorphisms detection) and cost-effective (approximately 4.3 USD per test) identification of pathogens [24]. RPA-based diagnostic tools for several economically important phytopathogens have been developed in recent years [26–40]. However, production and mass availability of affordably priced RPA assays that carry the possibility of multiple detections through the analysis of a single sample remains the single most formidable challenge for phytopathogen diagnostics at the consumer level [43]. A 'sample-in-answer-out' device purifies and digitalizes SARS-CoV-2 RNA for reverse-transcriptase (RT) RPA-based pathogen detection in a fully automated manner [44]. Such technology integrating paper- or chip-based portable microfluidic devices and RPA workflow can be appropriately modified for detecting mixed infections in point-of-care and point-of-need testing. Likewise, CRISPR/Cas in combination with integrated RPA/LFD devices (Bio-SCAN; biotin-coupled specific CRISPR-based assay for nucleic acid detection) provide a highly convenient and amenable technology for the detection of specific alleles in breeding lines, transgenic events, and rapid detection of fungal and bacterial pathogens [45].

Species belonging to the genus *Pantoea* are ubiquitous across several ecological niches, making rapid identification of *Pantoea* at the species or infraspecies level extremely difficult. First reported on Long Island in New York State in 1897, Stewart's wilt of corn has lately

emerged in several other parts of the world including Canada, Mexico, Austria, Argentina, Bolivia, Italy, Poland, Romania, Russia, China, Korea, Malaysia, Thailand, and Vietnam [46]. Additionally, *P. stewartii* subsp. *stewartii* introductions in native environments—through contaminated seed or alternative non-host plant material—cannot be ruled out, making surveillance certification and phytosanitary restrictions an essential clause for international trading. Timely disease management requires rapid and accurate identification of the disease-causing pathogen and subsequent roguing of the infected plant material from the field, thereby limiting the spread of disease. A low-cost and robust identification is also paramount for plant product health testing facilities, especially in a resource-limited setup such as the quarantine regulation ports. In this study, we report a reliable, specific, and sensitive RPA assay for the detection of the plant-pathogenic genus *Pantoea stewartii* subsp. *stewartii* currently regulated in more than 100 countries [47]. Although nonpathogenic on corn, *P. stewartii* subsp. *indologenes* may occasionally be present on corn seeds of tropical or subtropical origin as part of the resident bacterial population [22], thus warranting facile taxonomic discrimination between the two subspecies. To the best of our knowledge, the RPA assay described here provides the first molecular diagnostic tool for rapid, sensitive, and highly specific subspecies-level detection of *P. stewartii* subsp. *Stewartii* discriminating it from the subsp. *Indologenes*. Currently, unambiguous identification of the two *Pantoea stewartii* subsp. Remains time-consuming and expensive, relying on step-down PCR-based SNP profiles and MALDI–TOF MS protein fingerprints [22].

Pathogenicity of *P. stewartii* subsp. *stewartii* is associated with the biosynthesis of stewartan EPS, which causes xylem occlusion, vascular streaking, bacterial oozing, and the characteristic wilting of sweet corn. The tripartite stewartan biosynthetic system consists of the 12-gene *wceI* cluster, *wceII*, and *wceIII* genes [48]. RPA primers and probes were designed from the intergenic spacer region present between the *cpsA* and *cpsB* genes of the *wceI* operon (Figure 1). The *cpsA* and *cpsB* genes encode a glycosyltransferase WceG and a periplasmic protein Wza, respectively, that are involved in the biosynthesis and export of EPS. Alignment of the intergenic sequence between the *cpsA* and *cpsB* genes of *P. stewartii* subsp. *stewartii* (strain DC283, GeneBank acc. no. GCA_000248395.2) and *P. stewartii* subsp. *indologenes* (strain LMG2632, GenBank Acc. No. JPKO01000031) revealed a 182-base-pair deletion in *P. stewartii* subsp. *indologenes* strain LMG2632 [47].

The RPA assay reported here provides the first proof-of-concept for molecular detection and diagnosis based on the amplification of a unique genomic region differentiating the pathogenic and quarantine agent *P. stewartii* subsp. *stewartii* from nonpathogenic subsp. *indologenes*. *P. stewartii* subsp. *stewartii* can be detected in real-time using a portable isothermal nucleic acid detection system or a lateral flow dipstick. The integrated RPA/LFD assay is also amenable to minimal sample processing and performs well with high sensitivity using crude extracts from infected plants and incubated in a closed hand under field conditions. Current efforts in the lab are directed towards optimizing a multiplex assay using a cocktail of primer/probes for taxonomic differentiation of various species of *Pantoea* complex posing significant agricultural concern. The applicability of such assays for in-field diagnosis and in resource-limited quarantine laboratories cannot be over-emphasized.

**Author Contributions:** Concept: W.Z. and H.W.; methodology and investigation: L.C. and Q.T.; data analysis: L.C., Q.T., Q.M., X.B., P.X., J.L., W.Z. and H.W.; supervision and funding support: W.Z.; writing and editing: L.C., W.Z. and H.W. All authors have read and agreed to the published version of the manuscript.

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