

Article

Development and Application of a Loop-Mediated Isothermal Amplification (LAMP) Assay for the Detection of *Pseudomonas syringae* Pathovars *pisi* and *syringae*

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Abstract: *Pseudomonas syringae* causes bacterial blight (BB) disease worldwide on economically important fruit and vegetable crops including field pea (*Pisum sativum* L.). The two pathovars responsible for BB in field pea are *Pseudomonas syringae* pathovar *pisi* (Psp) and *syringae* (Pss). In the field, both pathovars cause indistinguishable symptoms on field pea and require laboratory diagnosis to determine the causal pathovar. To aid in-field and laboratory diagnosis, accurate, and robust loop-mediated isothermal amplification (LAMP) assays for Psp and Pss were developed. The assays were able to detect Psp or Pss on live or heat-killed bacterial cells, plant exudates, seeds, and DNA extracts with no inhibitory effects. The two specific LAMP assays developed detected Psp and Pss accurately in less than 20 min and no cross-reaction was observed with 18 strains of closely related species of *Pseudomonas syringae*. Compared to the conventional PCR assays, the two LAMP assays were equally specific but have advantages of producing quicker and visual live results, enabling early detection and differentiation of Psp and Pss. Our results suggested a potential use of LAMP assays for laboratory testing and can be applied for in-field surveys.

Keywords: bacterial blight; diagnostics; frost; field pea; epidemic; surveillance



Citation: Kant, P.; Fruzangohar, M.; Mann, R.; Rodoni, B.; Hollaway, G.; Rosewarne, G. Development and Application of a Loop-Mediated Isothermal Amplification (LAMP) Assay for the Detection of *Pseudomonas syringae* Pathovars *pisi* and *syringae*. *Agriculture* **2021**, *11*, 875. <https://doi.org/10.3390/agriculture11090875>

Academic Editor: Roy Kennedy

Received: 2 August 2021

Accepted: 9 September 2021

Published: 13 September 2021

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1. Introduction

Pseudomonas syringae is a gram-negative phytopathogenic bacterium within the gamma subgroup of the Proteobacteria, and up to 60 pathovars have been characterized based on their host range [1,2]. *Pseudomonas syringae* can be found both as a harmless epiphyte and as a plant pathogen, causing disease in most cultivated plant species [2]. In field pea (*Pisum sativum* L.), Bacterial Blight (BB) is caused by both *Pseudomonas syringae* pathovar *pisi* (Psp) and *Pseudomonas syringae* pv. *syringae* (Pss). Pss is more aggressive than Psp, affecting winter sown field pea in high severity frost affected areas worldwide [3,4] and causing epidemics that result in high yield losses [4,5]. The symptoms caused by Psp and Pss are often indistinguishable in the field. However, both pathovars have different pathogenicity mechanisms. Psp has a narrow host range of field pea only, has an epiphytic phase [6] and consists of eight currently identified races [7–10]. In contrast, Pss is a heterogenous pathovar whose members are collectively able to cause disease in >200 different plant species [11]. Pss isolates from field pea can be differentiated based on pathogenicity and virulence response on a host and non-host plants such as field pea, other legume crops and non-legumes crops. Molecular testing such as repetitive PCR, multi-locus sequence typing (MLST) and *syxB* gene also differentiate them. Pss isolates from field pea are highly virulent on field pea, suggesting a possible pathogenic specialization in this group [12].

Traditionally, characterization of the Psp and Pss involved morphological studies and biochemical tests such as LOPAT profiling [13,14] which was time-consuming and couldn't separate closely related isolates. Molecular testing such as endpoint PCR assays can distinguish between pathovars Psp [15] and Pss [16,17] accurately, but they require the processing of plant tissue either via DNA extraction or colony isolation, conducting a PCR reaction and gel electrophoresis. The application of one technology that has become increasingly popular in recent years for rapid laboratory and in-field detection of human, animal, and plant pathogens is loop-mediated isothermal amplification (LAMP) [18,19]. There are many advantages of LAMP over conventional PCR. LAMP is less sensitive to inhibitors. Therefore, it is adaptable for use with un-purified clinical and field samples [18].

In recent years, Pss has become the dominant pathogen causing BB of field peas in Australia [4,20] and outbreaks have become more frequent and destructive. To minimize the losses due to these outbreaks, the field pea breeding program actively breeds resistance against both Psp and Pss. Field pea surveys are conducted annually to determine the population dynamics of pathogens causing BB to inform the breeding program. Determining the causal pathogen associated with BB of field peas rapidly in-field would further aid the resistance breeding program. The objective of this study was to develop LAMP assays that could provide a rapid screening approach to detect and differentiate between Psp and Pss isolates in field pea and also provide the capability of in-field testing for the early detection of these pathogens. The approaches utilised in this study were (i) to design two separate LAMP assays for detection of Pss and Psp, (ii) validate these tests for both specificity and limit of detection (LOD), and (iii) evaluate the LAMP assays as a surveillance tool on field-collected plant and seed samples.

2. Materials and Methods

2.1. Bacterial Isolates

Bacterial isolates used in this study were sourced from culture collections of the Victorian Plant Pathology Herbarium (VPRI), Bundoora (Agriculture Victoria); and the Plant Pathology Herbarium (DAR), Department of Primary Industries, New South Wales (Table 1). The list contained 37 isolates each of Psp and Pss; 18 closely related isolates; and two *Pseudomonas viridiflava* (Pv) and one *Pseudomonas cichorii* (Pc). Pv and Pc were included in the study as an outgroup for genomic comparison and to validate LAMP assays. Fifty historical BB isolates from the Field Crops Pathology Laboratory at Horsham collections that were previously identified as Psp or Pss based on pathogenicity test on a bean, field pea and lemon stab according to Mazarei and Kerr 1990 [21] and maintained from the 1980s as a freeze-dried filter paper disc in vacuumed glass ampules, were revived and submitted to VPRI collection for use in this study.

Table 1. Description of isolates used for designing and validation of the loop-mediated isothermal amplification (LAMP) assays for the detection of *Pseudomonas syringae* pathovars *pisi* (Psp) and *Pseudomonas syringae* pathovars *syringae* (Pss).

Identity	Collection	Numbers	Year of Collection	Host Plant	Pathovar-Specific PCR	Time to Positive for Psp	Anneal Derivative	Time to Positive for Pss	Anneal Derivative	Reference
<i>P. cichorii</i>	VPRI	42,174	1986	<i>Lactuca sativa</i> (Lettuce)	ND	UD	UD	UD	UD	
<i>P. viridiflava</i>	VPRI	42,172	2003	<i>Pisum sativum</i> (Field Pea)	PEL	UD	UD	UD	UD	
<i>P. viridiflava</i>	VPRI	42,173	1987	<i>Ranunculus</i> sp. (Buttercup)	PEL	UD	UD	UD	UD	[22]
Psp Race2	VPRI	42,161	1944	<i>Pisum sativum</i> (Field Pea)	Psp	5.6	90.1	UD	UD	[22]
Psp Race2	VPRI	42,165	1992	<i>Pisum sativum</i> (Field Pea)	Psp	7.7	90.1	UD	UD	[23]
Psp Race3	VPRI	42,162	1975	<i>Pisum sativum</i> (Field Pea)	Psp	8.0	89.9	UD	UD	[22]
Psp Race3	VPRI	42,166	1992	<i>Pisum sativum</i> (Field Pea)	Psp	5.4	89.6	UD	UD	[20]
Psp Race4	VPRI	42,163	1975	<i>Pisum sativum</i> (Field Pea)	Psp	4.6	89.6	UD	UD	[22]
Psp Race6	VPRI	42,167	2003	<i>Pisum sativum</i> (Field Pea)	Psp	4.9	89.9	UD	UD	[20]
Psp Race6	VPRI	42,168	2001	<i>Pisum sativum</i> (Field Pea)	Psp	9.6	89.7	UD	UD	
Psp Race6	VPRI	42,170	1993	<i>Pisum sativum</i> (Field Pea)	Psp	8.0	89.8	UD	UD	
Psp Race6	VPRI	42,171	1993	<i>Pisum sativum</i> (Field Pea)	Psp	9.6	89.7	UD	UD	[23]
Psp Race7	VPRI	42,164	NA	<i>Pisum sativum</i> (Field Pea)	Psp	9.2	89.7	UD	UD	[3]
Pss	VPRI	42,146	1981	<i>Phaseolus vulgaris</i> (French Bean)	Pss	UD	UD	5.5	90.7	
Pss	VPRI	42,147	1978	<i>Prunus domestica</i> (Plum)	Pss	UD	UD	3.7	90.3	
Pss	VPRI	22,505	NA	<i>Prunus domestica</i> (Rosaceae)	Pss	UD	UD	4.6	90.1	
Pss	VPRI	32,448	NA	<i>Impatiens</i> sp. [Balsaminaceae]	Pss	UD	UD	4.8	91.7	
Pss	VPRI	42,148	1983	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	4.8	90.5	[21]
Pss	VPRI	42,149	1984	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	5.7	90.7	[21]
Pss	VPRI	42,150	2003	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	3.8	90.7	
Pss	VPRI	42,143	2012	<i>Phaseolus vulgaris</i> (French Bean)	Pss	UD	UD	6.7	90.7	[20]
Pss	VPRI	42,151	2003	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	5.6	90.7	[20]
Pss	VPRI	42,607	2014	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	5.6	90.6	
Pss	VPRI	21,225	1996	<i>Prunus persica</i> [Rosaceae]	Pss	UD	UD	5.7	90.5	
Pss	VPRI	42,827	2016	<i>Vicia faba</i> [Fabaceae]	Pss	UD	UD	4.6	90.6	
Pss	VPRI	41,526	1999	<i>Brassica oleracea</i> [Brassicaceae]	Pss	UD	UD	5.6	90.7	
Pss	VPRI	32,662	2006	<i>Grevillea</i> sp. [Proteaceae]	Pss	UD	UD	8.5	90.7	

Table 1. Cont.

Identity	Collection	Numbers	Year of Collection	Host Plant	Pathovar-Specific PCR	Time to Positive for Psp	Anneal Derivative	Time to Positive for Pss	Anneal Derivative	Reference
<i>Pseudomonas marginalis</i>	VPRI	32,782	2006	<i>Pastinaca sativa</i> [Apiaceae]	ND	UD	UD	UD	UD	
<i>Pseudomonas fluorescens</i>	VPRI	41,564	1996	<i>Lactuca sativa</i> [Asteraceae]	ND	UD	UD	UD	UD	
<i>Pseudomonas fluorescens</i>	VPRI	41,213	2007	<i>Beta vulgaris</i> [Chenopodiaceae]	ND	UD	UD	UD	UD	
<i>Pseudomonas corrugata</i>	VPRI	41,570	1997	<i>Solanum lycopersicum</i> [Solanaceae]	ND	UD	UD	UD	UD	
<i>Pseudomonas marginalis</i>	VPRI	32,683	2006	<i>Hesperis matronalis</i> [Brassicaceae]	ND	UD	UD	UD	UD	
<i>Pseudomonas syringae</i> pv. <i>coriandricola</i>	VPRI	41,569	2003	<i>Coriandrum sativum</i> [Apiaceae]	ND	UD	UD	UD	UD	
<i>Pseudomonas putida</i>	VPRI	32,399	2002	<i>Solanum tuberosum</i> [Solanaceae]	ND	UD	UD	UD	UD	
<i>Pseudomonas agarici</i>	DAR	41,321	1982	<i>Agaricus bisporus</i> (Lange) Imbach	ND	UD	UD	UD	UD	
<i>Pseudomonas pickettii</i>	DAR	65,900	NA	Clavicipitaceae	ND	UD	UD	UD	UD	
<i>Pseudomonas aeruginosa</i>	DAR	41,354	1983	Clavicipitaceae	ND	UD	UD	UD	UD	
<i>Pseudomonas tolaasii</i>	DAR	35,636	NA	Clavicipitaceae	ND	UD	UD	UD	UD	
<i>Pseudomonas chlororaphis</i>	DAR	76,122	2001	<i>Brassica napus</i> L. var <i>napus</i>	ND	UD	UD	UD	UD	
<i>Xanthomonas campestris</i>	VPRI	32,436	NA	NA	ND	UD	UD	UD	UD	
<i>Xanthomonas campestris</i>	VPRI	41,521	1997	<i>Eriostemon</i> sp.	ND	UD	UD	UD	UD	
<i>Erwinia chrysanthemi</i>	VPRI	42,393	1998	<i>Zingiber officinale</i> Roscoe	ND	UD	UD	UD	UD	
<i>Erwinia chrysanthemi</i>	VPRI	42,392	1978	<i>Solanum tuberosum</i> L.	ND	UD	UD	UD	UD	
<i>Ralstonia solanacearum</i>	VPRI	42,679	2015	<i>Solanum tuberosum</i> L.	ND	UD	UD	UD	UD	
<i>Ralstonia solanacearum</i>	VPRI	42,677	2015	<i>Solanum tuberosum</i> L.	ND	UD	UD	UD	UD	
Pss	VPRI	43,444	2007	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	7.4	90.2	
Pss	VPRI	43,445	2007	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	7.4	90.6	
Pss	VPRI	43,446	2007	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	7.4	90.5	
Pss	VPRI	43,449	1990	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	8.2	90.7	
Pss	VPRI	43,450	1983	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	17.4	90.3	
Pss	VPRI	43,451	2007	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	10.3	90.5	
Pss	VPRI	43,452	1989	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	8.4	90.5	
Pss	VPRI	43,453	1989	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	10.4	90.5	
Pss	VPRI	43,454	2007	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	8.1	90.7	
Pss	VPRI	43,455	1989	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	7.9	90.7	
Pss	VPRI	43,456	1989	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	8.3	90.7	
Pss	VPRI	43,457	1989	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	10.3	90.6	
Pss	VPRI	43,458	1989	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	7.5	90.5	
Pss	VPRI	43,460	1989	<i>Phaseolus vulgaris</i> (French Bean)	Pss	UD	UD	7.5	90.4	
Pss	VPRI	43,461	1991	NA	Pss	UD	UD	8.0	90.5	
Pss	VPRI	43,462	1989	NA	Pss	UD	UD	5.7	90.4	
Pss	VPRI	43,463	1991	NA	Pss	UD	UD	8.8	90.5	
Pss	VPRI	43,464	1991	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	8.1	90.3	
Pss	VPRI	43,465	1991	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	8.6	90.5	
Pss	VPRI	43,466	2007	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	10.4	90.5	
Pss	VPRI	43,467	1991	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	8.6	90.4	
Pss	VPRI	43,498	1991	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	8.4	90.5	
Pss	VPRI	43,482	1991	<i>Pisum sativum</i> (Field Pea)	Pss	UD	UD	6.8	90.4	
Psp	VPRI	43,468	1991	<i>Pisum sativum</i> (Field Pea)	Psp	11.4	89.8	UD	UD	
Psp	VPRI	43,475	1991	<i>Pisum sativum</i> (Field Pea)	Psp	5.6	89.7	UD	UD	
Psp	VPRI	43,476	1991	<i>Pisum sativum</i> (Field Pea)	Psp	6.5	89.7	UD	UD	
Psp	VPRI	43,477	2007	<i>Pisum sativum</i> (Field Pea)	Psp	5.8	89.9	UD	UD	
Psp	VPRI	43,478	1991	<i>Pisum sativum</i> (Field Pea)	Psp	6.0	89.9	UD	UD	
Psp	VPRI	43,479	1991	<i>Pisum sativum</i> (Field Pea)	Psp	6.5	89.6	UD	UD	
Psp	VPRI	43,480	1991	<i>Pisum sativum</i> (Field Pea)	Psp	6.2	90.1	UD	UD	
Psp	VPRI	43,481	1991	<i>Pisum sativum</i> (Field Pea)	Psp	6.2	90.1	UD	UD	
Psp	VPRI	43,483	1991	<i>Pisum sativum</i> (Field Pea)	Psp	11.3	90.1	UD	UD	
Psp	VPRI	43,484	1991	<i>Pisum sativum</i> (Field Pea)	Psp	6.1	90.1	UD	UD	
Psp	VPRI	43,485	1991	<i>Pisum sativum</i> (Field Pea)	Psp	6.3	89.9	UD	UD	
Psp	VPRI	43,486	1991	<i>Pisum sativum</i> (Field Pea)	Psp	6.3	89.6	UD	UD	
Psp	VPRI	43,487	1991	<i>Pisum sativum</i> (Field Pea)	Psp	6.4	89.6	UD	UD	
Psp	VPRI	43,488	1991	<i>Pisum sativum</i> (Field Pea)	Psp	7.5	89.9	UD	UD	
Psp	VPRI	43,489	1991	<i>Pisum sativum</i> (Field Pea)	Psp	6.1	89.7	UD	UD	
Psp	VPRI	43,490	1991	<i>Pisum sativum</i> (Field Pea)	Psp	5.7	89.8	UD	UD	
Psp	VPRI	43,447	2007	<i>Pisum sativum</i> (Field Pea)	Psp	5.7	89.7	UD	UD	
Psp	VPRI	43,448	1983	<i>Pisum sativum</i> (Field Pea)	Psp	5.7	89.8	UD	UD	
Psp	VPRI	43,491	1991	<i>Pisum sativum</i> (Field Pea)	Psp	7.9	89.7	UD	UD	
Psp	VPRI	43,492	1991	<i>Pisum sativum</i> (Field Pea)	Psp	7.4	90.1	UD	UD	
Psp	VPRI	43,493	1991	<i>Pisum sativum</i> (Field Pea)	Psp	7.3	90.1	UD	UD	
Psp	VPRI	43,494	1991	<i>Pisum sativum</i> (Field Pea)	Psp	12.9	89.8	UD	UD	
Psp	VPRI	43,495	2007	<i>Pisum sativum</i> (Field Pea)	Psp	6.7	90.1	UD	UD	
Psp	VPRI	43,496	1991	<i>Pisum sativum</i> (Field Pea)	Psp	8.5	90.0	UD	UD	
Psp	VPRI	43,497	1991	<i>Pisum sativum</i> (Field Pea)	Psp	7.5	89.9	UD	UD	
Psp	VPRI	43,499	1991	<i>Pisum sativum</i> (Field Pea)	Psp	8.5	89.9	UD	UD	
Psp	VPRI	43,500	1991	<i>Pisum sativum</i> (Field Pea)	Psp	7.3	89.8	UD	UD	

The identity of isolates listed as Psp and Pss was confirmed with pathovar-specific PCR tests that target AN3 and AN7 for *Pseudomonas syringae* pathovar *pisi* (Psp) [15] and *syrB* for *Pseudomonas syringae* pathovar *syringae* (Pss) [16]. For *P. viridiflava*, PEL primers were used [22]. ND (not done), NA (not available) UD (Undetected). VPRI- Plant Pathology Herbarium of Victoria, AgriBio, Bundoora, Victoria. DAR-Plant Pathology Herbarium, Department Primary Industries, New South Wales.

In this study, the identity of isolates listed as Psp and Pss was confirmed with pathovar-specific PCR, targeting AN3/AN7 [15] and *syrB* [16] genes, respectively. All bacterial isolate cultures were initiated from single-colonies and maintained by monthly transfers on King's B medium [23] at 28 °C for 3 to 5 days and stored long term at −80 °C in 20% glycerol in nutrient yeast broth (NYB).

2.2. Bioinformatics Pipeline for LAMP Primer Design

The unique genomic regions used for the Psp LAMP primer design were identified by whole-genome sequence data comparison of 23 isolates of the *Pseudomonas syringae* species complex. Ten isolates of Psp, comprising of Race 2 (VPRI 42161 and 42165), Race 3 (VPRI 42162), Race 4 (VPRI 42163), Race 6 (VPRI 42167, 42168, 42170, and 42171) and Race 7 (VPRI 42164) and ten isolates of Pss from different hosts (VPRI 22505, 32448, 42143, 42146, 42147, 42148, 42149, 42150, 42151, and 42607) as well as two isolates of *Pseudomonas viridiflava* (Pv) (VPRI 42172 and 42173), and one isolate of *Pseudomonas cichorii* (Pc), VPRI 42174, were included for comparison (Table 1). Firstly, the 10 sequences of the Psp were compared using the pairwise alignment tool Lastz, (www.bx.psu.edu/~rsharris/lastz/, 17 May 2021) [24] for extraction of the common sequences. These sequences were compared with the corresponding concatenated sequence from 10 Pss isolate sequences and three outgroup isolates sequences. The pipeline for the alignment and genome comparison has been submitted to GitHub (<https://github.com/Pragya2019/Lamp->, 17 May 2021). Further, the sequences were compared to the NCBI public non-redundant nucleotide database using BLASTn [25] to ensure sequence specificity to Psp and not with other bacteria.

Pss-specific LAMP primers were designed using conserved sequences from the *sala* gene based on the previous work of Busot et al. (2015) [26] as it is associated with the regulation of syringomycin production explicit to Pss [27]. This region was identified in the Pss Type strains B301D and B728a with a single amino acid change between them and compared using BLASTn algorithm [25] to all sequences of Pss isolates sequenced in this study (see above).

Psp and Pss-specific LAMP primers were designed using PrimerExplorerV5 in <http://primerexplorer.jp/lampv5e/index.html> (accessed date 17 May 2021) following the default settings. Each primer set consisted of forward outer (F3), backward outer (B3), forward inner (FIP), backward inner (BIP), loop forward (LF) and loop backward (LB) primers (Table 2). The primers were synthesized from Sigma–Aldrich (Castle Hill, NSW, Australia).

Table 2. Details of LAMP primers for *Pseudomonas syringae* pathovar *pisi* and *Pseudomonas syringae* pathovar *syringae*.

Primers	Sequence (5' to 3')	Nucleotide Start Position	Nucleotide End Position
<i>Pseudomonas syringae</i> pathovar <i>pisi</i> sequence submitted to GenBank accession no. MZ043759			
Psp_F3	CTCGTGCACGATTCACCAT	198	219
Psp_B3	CGCCTTACTCATTAGTACGCTAA	426	488
Psp_FIP *	(F1c) CGTCTACAGCTAATCAT-GCCTCT	278	300
	(F2) AGTCTCCTACCTCTATC-CGTCAT	227	248
Psp_BIP *	(B1c) TAGCGCTTTCGCACAGCCT	316	334
	(B2) AGGCATCTTGCTCCACGCAT	393	422
Psp_LF	TGCTCCACGTGGGTTCACAG	253	272
Psp_LB	GAGGCCATGAGTGGGCTGAGA	363	383
<i>Pseudomonas syringae</i> pathovar <i>syringae</i> to GenBank accession no. MZ043758			
Pss_F3	CATCCTTGACCTCCGAAGCG	148	167
Pss_B3	CAAGCCATCGAGGCCAATGG	401	420
Pss_FIP *	(F1c) CACCGCGCCG-TAGTTGAACCT	262	282
	(F2) ATGCGCAACG-GTTGATGGGTA	173	193
Pss_BIP *	(B1c) TACCTTGCCGATGTGCT-GCGCA	310	331
	(B2) ATTGCAACGGTAGTCGCCA	381	399

Table 2. Cont.

Primers	Sequence (5' to 3')	Nucleotide Start Position	Nucleotide End Position
Pss_LF	ATCAGCCCTGTACGACTCGCTC	210	231
Pss_LB	GCATCGGCAAATTTGAATGTCC	359	380

* A forward inner primer (FIP) consisted of the complementary sequence of F1c and F2, and the backward inner primer (BIP) consisted of B1c and complementary sequence of B2.

2.3. LAMP Assay Validation

Initially, conventional PCR reactions using the outer primers, F3 and B3, of each LAMP primer set were run on two representative isolates, Psp (VPRI 42167) and Pss (VPRI 42143), and the resulting amplicons were sequenced to confirm the correct DNA target region was being amplified. Colony PCR reactions totalled 25 µL consisting of 12.5 µL MyTaq™ master mix (Bioline), 1 µL forward (F3) and reverse (B3) primers at a concentration of 1 µM each. Templates were prepared from 2 µL of heat-killed bacterial cells (concentration at the O.D of 0.2) and were run with the following conditions; 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 63 °C, and 30 s at 72 °C, and a final extension cycle at 72 °C for 5 min. Amplification of the correctly sized PCR products for Psp and Pss (251 and 273 bp, respectively) was confirmed by gel electrophoresis on 2% *w/v* agarose gel at 100 amp volt for 40 min. The target specificity of these PCR products was confirmed by Sanger sequencing.

LAMP reactions were done in a portable LAMP machine, Genie III® (OptiGene) using the assay profile of 65 °C for 30 min followed by melting curve analysis step (98–80 °C ramping at 0.1 per/min) for 10 min, or in a QuantStudio™ 3 Real-Time PCR machine (Thermo Fisher Scientific) creating the same profile with 120 cycles (30 min) at 65 °C and an in-built melt, was added at the end at 98 °C at 1.6 °C/s. LAMP reactions consisted of 20 µL volume containing 2 µL of the bacterial DNA sample, 10 µL GspSSD2.0 Isothermal Master Mix iso-004 buffer (OptiGene Ltd., West Sussex, UK), 0.2 µM each F3/B3 primer, 1.6 µM each FIP/BIP primer, 0.8 µM each LF/LB primer and 6 µL of water. A positive amplification plot showed an 'S' shaped sigmoid curve reflecting the increase in fluorescence detected and negative results stayed relatively flat. The amplification time or Time to positive (Tp) was determine in mins. The temperature of the anneal curve (Tm) was also recorded, indicating the product specificity (Figure 1).

2.3.1. Specificity Analysis

The specificity of the two LAMP assays was evaluated using 95 isolates of plant-associated bacteria (Table 1). Specifically, 37 isolates of Psp, 37 isolates of Pss and 18 isolates of closely related *Pseudomonas* species and other bacteria were tested to validate the Psp and Pss LAMP assays. Single bacterial colonies were diluted in 100 µL of water and a 2 µL sample of the bacterial suspensions were directly tested or heat-treated at 95 °C for 10 min and detection was done in the Genie III or QuantStudio™ PCR machine as previously described.

2.3.2. Limit of Detection

To determine the sensitivity of the LAMP assays, a 10-fold serial dilution (1×10^0 to 1×10^{-6}) of bacterial cultures of Psp (VPRI 42167) and Pss (VPRI 42143) was prepared from a bacterial suspension of each isolate with an optical density of 0.2 (equivalent to 6.5×10^8 CFU/mL) and tested using Psp and Pss LAMP assays as described above. One hundred microlitres of each serially diluted bacterial cell suspension were cultured in duplicate on KB media [23] and incubated for 48 h at 25 °C to determine the number CFU/mL.

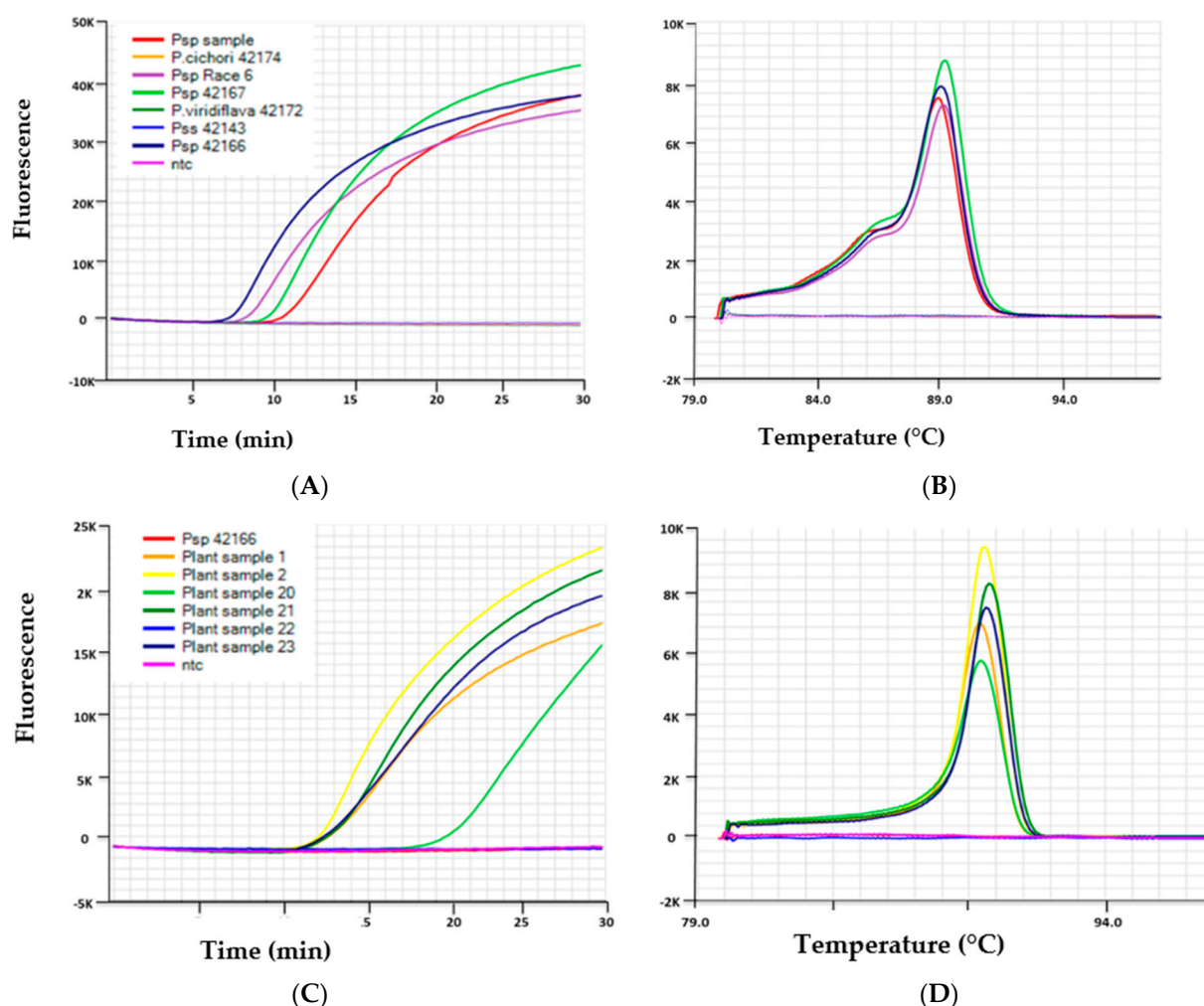


Figure 1. Results of LAMP assays shown as amplification curves (A,C) and melt curves (B,D) performed on Genie[®] III with representative samples. (A): The Psp LAMP assay generated positive amplification curves from Psp isolates VPRI 42166 (Race 3), VPRI 42167 (Race 6), Psp plant sample, and no amplification (depicted by a flat line) from isolates of Pss VPRI 42143, *P. viridiflava* VPRI 42172 and *P. cichorii* VPRI 42174. (B): Melt curves produced anneal derivative from the positive samples of Psp with a range of 89.6–90.1 which is indicative of a positive result for this assay. (C): The Pss LAMP assay generated positive amplification curves from Pss positive plant samples 1, 2, 20, 21 and 23 and no amplification from Psp 42166 and ntc. (D): Melt curves produced from the positive samples of Pss with an anneal derivative range of 90.1–90.7, which is indicative of a positive result for this assay.

2.4. LAMP as Surveillance and Diagnostic Tool

2.4.1. Early Detection of Bacterial Blight

To determine if a LAMP assay could detect the presence of Psp or Pss before the visual symptoms of BB appear on plants, an experiment was designed using the BB susceptible field pea variety Kaspera inoculated with Psp (VPRI 42166) and Pss (VPRI 42607) separately in the glasshouse conditions using the bacterial inoculation protocol described by Rodda et al. (2015) [20]. In brief, the experiment was designed in three replicates with three plants per pot and spray inoculated with a bacterial suspension of optical density of $A_{600} = 0.2$ supplemented with 0.05% *v/v* Pulse[®] Penetrant (Nufarm, Australia) as a surfactant. Plants were kept under misting for 5 min every 3 h for 3 days. Two leaves per plant from each replicate were taken each day from day one to seven post-inoculation or until visual symptoms were obvious. Plant tissues were washed with mild soap and rinsed three times with sterilized water before incubated overnight in 5 mL of sterilized water in a 4 °C fridge and 2 μ L suspension was tested with the LAMP assays as described above.

The non-inoculated and plants inoculated with water containing surfactant were used as a negative control.

2.4.2. Seed Testing

Seeds of BB susceptible variety Kaspera and the moderately BB resistant variety PBA Percy were used to examine if the LAMP assay could detect bacteria in BB infected seed. The seeds were sourced from naturally infected field peas from the Plant Breeding Center, Horsham in 2019. One hundred thrashed seeds were submerged in 300 mL of sterile phosphate saline buffer pH 7.0 in a 500 mL Schott bottle for 24 h at a cool temperature below 20 °C. The homogenate was serially diluted to 10^{-3} in sterile phosphate saline, and each dilution was tested using the Psp and Pss LAMP assays and plated out in triplicate on the semi-selective media containing sucrose-nutrient agar supplemented with boric acid, cefuroxime, cycloheximide and cephalixin [28]. The plates were incubated for 48 h at 25 °C and assessed for colony growth. A random 10 colonies were tested from each plate with the Psp and Pss-specific LAMP assays as described above.

2.4.3. Field Sample Testing

The two LAMP assays were applied on field pea plants from the field to identify which pathogen, Psp and/or Pss, was associated with the disease. In 2019, naturally infected 50 leaf samples from a field trial comprising 50 varieties were collected. Infected leaves and stems were washed with mild soap, rinsed three times with RO (reverse osmosis) water before wrapped with paper towels and kept in a plastic bag to store in a fridge and processed within one week. Two to three symptomatic leaves were submerged in 5 mL sterile distilled water overnight at 4 °C and 2 µL of the exudate suspension was directly used as a test for each sample using the Psp and Pss LAMP assays. Bacteria were also isolated from the infected leaves by plating on a semi-selective media. The colonies were tested as Psp or Pss and confirmed with PCR as described above.

3. Results

3.1. Primer Design and Development of the LAMP Assays

The Lastz alignment of all Psp isolates in this study identified a highly conserved sequence region of 507 bp, which was not identified in Pss isolates and closely related species of *Pseudomonas* species. The Blastn analysis indicated that this region shared 99.61% nucleotide identity with Psp strain PP1 [29] genome accession number NZ_CP034078.1 and nucleotide region of 1736718-1737224 bp. This region encodes for a 92 amino acid hypothetical protein GenBank accession RML51337.1. This 507 bp sequence was used for the primer design.

A 2347 bp region of the *salA* gene (Genbank number AF02280) from Pss strain B728a (Genbank accession N_007005.1) was conserved to all Pss. An 809 bp region spanning from nucleotide 3052738-3053546 (Genbank accession N_007005.1) showed 100% sequence similarity among the 10 Pss isolates sequenced in this study. This region was targeted for Pss-specific LAMP primer design using the custom Blastn comparisons in Geneious Prime (Auckland, New Zealand).

LAMP outer primers Psp_F3 and Psp_B3 (Table 2) amplified a 251 bp region when tested with Psp isolate (VPRI 42167), and amplification was observed when Pss isolate (VPRI 42143) was tested. Similarly, the outer primers Pss_F3 and Pss_B3 primers amplified a band of 273 bp when tested with bacterial cells of Pss isolate (VPRI 42143) and did not amplify any band with Psp isolates. Specificity was also confirmed by direct sequencing of the PCR products. The broader regions used for the LAMP assays primer designs were submitted to the GenBank with the accession number MZ043758 for Psp and MZ043758 for Pss.

3.2. Specificity of the LAMP Assays

The LAMP assays for Psp and Pss were highly specific and did not show any amplification with closely related *Pseudomonas* species strains including Pv, Pc, *P. corrugate*, *P. fluorescens*, *P. marginalis*, *P. putida*, *P. flectens*, *P. agarici*, *P. aeruginosa*, *P. pickettii*, *P. chlororaphis*, *P. tolaasii*, as well as *P. syringae* pathovar *coriandricola* (Table 1). No amplification was observed for other bacterial species tested including *Xanthomonas campestris*, *Erwinia chrysanthemi*, and *Ralstonia solanacearum*.

Results for the Psp and Pss LAMP assays correlated with results of the specific PCR assays targeting the AN7 fragment (Psp) and *syfB* (Pss). The Psp specific LAMP generated positive amplification curves for all 37 Psp isolates included in this study but did not amplify any of the Pss or other bacterial isolates. A positive amplification curve was confirmed with an anneal derivative ranging from 89.6–90.1 for each positive sample. The Pss specific LAMP generated positive amplification curves for all 37 Pss isolates included in this study but did not amplify any of the Psp or other bacterial isolates. A positive amplification curve was confirmed with an anneal derivative ranging from 90.1–90.7 for each positive sample (Table 1). The Pss LAMP assay also detected Pss that were isolated from hosts other than field pea (Table 1).

3.3. Limit of Detection

Specific LAMP assays for both Psp and Pss were highly sensitive achieving a limit of detection of 2.5×10^2 CFU/mL for isolate Psp (VPRI 42167) and 1.7×10^2 CFU/mL for Pss (VPRI 42143), respectively. The limit of detection was calculated by correlation of CFU concentration per micro litres and the number of PCR cycles required for serial dilution of Psp ($R^2 = 0.9786$) and Pss ($R^2 = 0.9587$) using the QuantStudio™ real-time PCR (Figure 2).

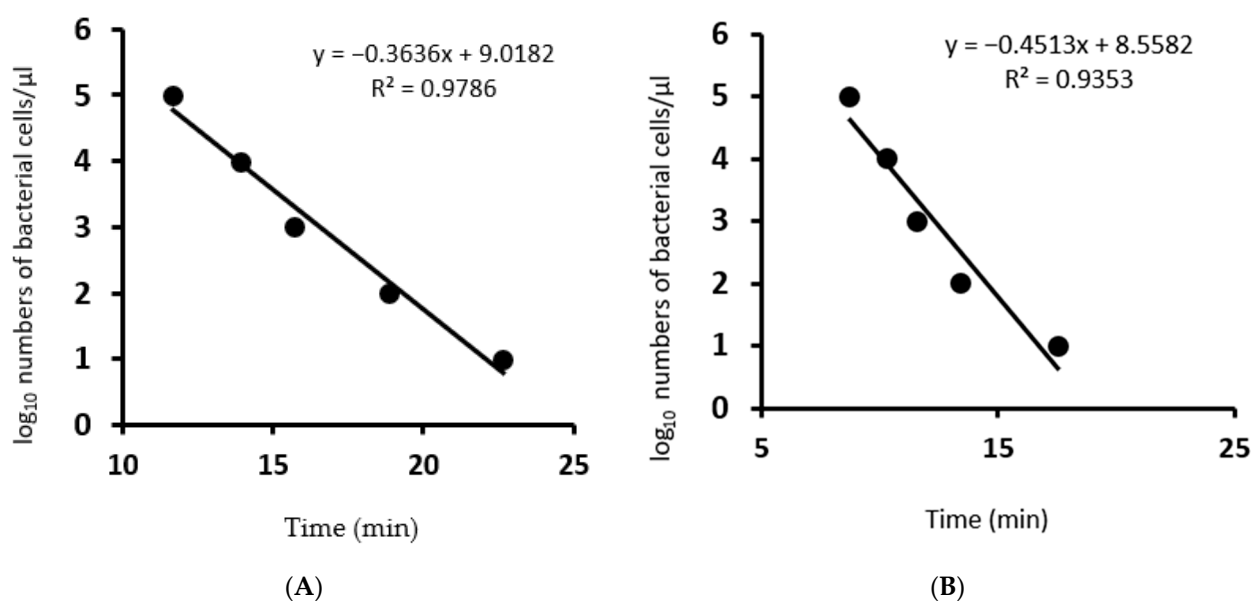


Figure 2. Regression curves generated in the LAMP assays for serial dilutions of live bacterial culture. Time to positive (Tp) is plotted against the log₁₀ bacterial cells μL^{-1} . (A): *Pseudomonas syringae* pathovar *pisi* primers, (B): *Pseudomonas syringae* pathovar *syringae* primers.

3.4. LAMP as Surveillance and Diagnostic Tool

3.4.1. Early Detection of Bacterial Blight

A time-course experiment showed the Psp- and Pss-specific LAMP assays were able to detect the presence of Pss and Psp in planta before the visual symptoms became obvious. Precautions were taken to use washed leaves so that the bacteria multiplying in the plant cells were detected. LAMP assays detected the presence of bacteria as early as four- and three days post-inoculation for Psp and Pss, respectively (Table 3). The Time to positive

(Tp) was gradually decreased from 27 min to 10 min from three days to ten days post-infection, respectively for Psp. The plants infected with Pss had lower Tp as compared to the plants infected with Psp on each day after inoculation. Although there were no significant differences in the Tp between the days after inoculation, an average time for the amplification in the LAMP assays decreased with the increase in the days after inoculation (Table 3), suggesting the increase in bacteria multiplying in the plant. The initial visual symptoms of water soaking developed in seven days post-inoculation for both pathogens and took 10 days to develop obvious symptoms of BB where the infected tissues became pale and started to develop necrosis. No amplification was observed from either test when leaves from uninoculated negative control plants or plants treated with surfactant alone were tested.

Table 3. Early detection of *Pseudomonas syringae* pathovar *pisi* (Psp) and *Pseudomonas syringae* pathovar *syringae* (Pss) from the inoculated field pea variety PBA Kasper before the visual symptoms appeared.

	Inoculated									Controls	
	DAI 2	DAI 3	DAI 4	DAI 5	DAI 6	DAI 7	DAI 8	DAI 9	DAI 10	Surfactant	Un-inoculated
<i>Pseudomonas syringae</i> pathovar <i>pisi</i>											
Tp (min)	*UD	27.32 ± 3.1	21.61 ± 2.2	19.84 ± 3.7	17.00 ± 2.5	14.54 ± 3.9	13.97 ± 2.0	12.07 ± 1.5	9.55 ± 2.1	UD	UD
AD (°C)	-	90.0	90.2	89.5	90.1	90.1	90.0	90.0	90.0	-	-
<i>Pseudomonas syringae</i> pathovar <i>syringae</i>											
Tp (min)	21.75 ± 5.1	19.79 ± 3.2	19.22 ± 2.7	17.28 ± 2.1	15.60 ± 3.1	15.19 ± 2.2	12.57 ± 3.8	12.19 ± 2.8	7.035 ± 1.1	UD	UD
AD (°C)	90.1	90.5	90.0	90.3	90.2	90.0	90.3	90.0	90.1	-	-

Plants were inoculated with Psp and Pss isolates separately. Samples were taken each day after inoculation (DAI). Tp (Time to positive), AD (anneal derivative), UD (undetected).

3.4.2. Detection of Bacteria from Seeds

The serial dilution of 10^{-1} , 10^{-2} and 10^{-3} of the seed suspensions showed an average Tp from 12 ± 5.1 , 14 ± 2.9 and 17 ± 2.5 min in Pss LAMP assays. Seed suspensions of 10^{-3} had an average of 373 ± 35 CFU mL $^{-1}$ when isolates from Kasper were plated, whereas the bacterial suspension from PBA Percy had and 244 ± 15 CFU mL $^{-1}$. All representative colonies tested were positive in the Pss LAMP assay and negative in the Psp LAMP assay.

3.4.3. Field Sample Testing

As a surveillance tool, the two LAMP assays were applied on the 50 BB naturally infected field pea varieties from the historical variety trial PHIST19. All the samples were found to be infected with Pss and not with Psp (Table 4). There was variability in the Tp recorded, ranging from 5.4 to 22.6. Interestingly, the susceptible Kasper had, on average, a lower Tp than other field pea varieties suggesting higher bacterial colonies. The colonies isolated from these samples were confirmed with *sydB* gene amplification in a PCR as Pss.

Table 4. Naturally infected bacterial blight samples of field pea collected from PHIST19 variety trial at Horsham for the detection *Pseudomonas syringae* pathovar *pisi* (Psp) and *Pseudomonas syringae* pathovar *syringae* (Pss) with the LAMP assays.

Year of Release	Field Pea Variety	Psp LAMP	Pss LAMP Tp (min)	Bacteria Isolated	Pathovar Specific PCR
1985	Wirrega	UD	7.5	Yes	Pss
1996	Bohatyar	UD	11.4	Yes	Pss
1993	Jupiter	UD	8.5	Yes	Pss
2000	Mukta	UD	9.4	Yes	Pss
2010	PBA Twilight	UD	8.6	Yes	Pss

Table 4. Cont.

Year of Release	Field Pea Variety	Psp LAMP	Pss LAMP Tp (min)	Bacteria Isolated	Pathovar Specific PCR
2003	Moonlight	UD	12.5	Yes	Pss
1998	Magnet	UD	15.6	Yes	Pss
2003	Sturt	UD	10.5	Yes	Pss
1985	Alma	UD	8.9	Yes	Pss
1992	Bonzer	UD	11.5	Yes	Pss
2000	Cooke	UD	12.8	Yes	Pss
1979	Whero	UD	17.8	Yes	Pss
2000	Snowpeak	UD	19.5	Yes	Pss
1995	Laura	UD	14.7	Yes	Pss
2002	Dunwa	UD	18.9	Yes	Pss
2000	Excel	UD	16.9	Yes	Pss
1964	Derrimut	UD	19.0	Yes	Pss
1920	White Brunswick	UD	20.1	Yes	Pss
2000	Soupa	UD	13.8	Yes	Pss
1998	Parafield	UD	17.4	Yes	Pss
1985	Glenroy	UD	8.0	Yes	Pss
2002	Kaspa	UD	5.8	Yes	Pss
2010	PBA Oura	UD	19.3	Yes	Pss
1893	Dun	UD	11.3	Yes	Pss
2014	PBA Pearl	UD	22.6	Yes	Pss
2011	PBA Percy	UD	12.5	Yes	Pss
1989	Diakum	UD	14.2	Yes	Pss
1999	Helena	UD	17.8	Yes	Pss
2013	PBA Wharton	UD	10.9	Yes	Pss
2000	Paravic	UD	11.8	Yes	Pss
1998	King	UD	8.9	Yes	Pss
2000	Santi	UD	5.4	Yes	Pss
1970	Dundale	UD	6.7	Yes	Pss
1992	Bluey	UD	5.7	Yes	Pss
2000	Yarrum	UD	12.8	Yes	Pss
1939	Colligian	UD	17.8	Yes	Pss
2002	Kiley	UD	12.5	Yes	Pss
1986	Maitland	UD	14.6	Yes	Pss
2011	Maki	UD	12.6	Yes	Pss
1998	Morgan	UD	13.5	Yes	Pss
2002	Kaspa	UD	6.5	Yes	Pss
2014	PBA Pearl	UD	19.8	Yes	Pss
2017	PBA Butler	UD	18.0	Yes	Pss
2010	PBA Ganyah	UD	5.6	Yes	Pss
2011	PBA Percy	UD	16.7	Yes	Pss
2002	Kaspa	UD	7.8	Yes	Pss
1939	Collegian	UD	10.3	Yes	Pss
1976	Cressy Blue	UD	10.7	Yes	Pss
2010	PBA Oura	UD	15.6	yes	Pss
2017	PBA Butler	UD	17.1	Yes	Pss

All isolates were reconfirmed with pathovar-specific PCR tests for *Pseudomonas syringae* pathovar *pisi* [15] (Psp) and *Pseudomonas syringae* pathovar *syringae* [16] (Pss). UD: Undetermined. Tp: Time to positive.

4. Discussion

The LAMP assays described here are rapid and reliable detection methods for Pss and Psp from field pea in-field and in the laboratory. The LAMP assays worked efficiently on the BB infected plant and seed suspensions without requiring any specialised equipment for sample preparation. This enabled testing on-farm, thus obviating the extra cost and time utilised for DNA extraction kits or plating in the laboratory. The LAMP assays were shown to be extremely sensitive, with a detection limit of 2.5×10^2 CFU mL⁻¹ for the Psp-specific assay and 1.7×10^2 CFU mL⁻¹ for the Pss-specific assay directly from a bacterial suspension. If the sample was identified as Psp, no cross-reactivity was observed with the

Pss LAMP assay and vice versa. This provided confidence in applying these LAMP tests to support large surveillance programs for rapid testing. Both pathovars were detected *in-planta* from infected samples within 5 to 20 min using the LAMP assays, which clearly demonstrates the utility of this method as an accurate, consistent, efficient and simple in-field assay when compared to existing lab-based PCR protocols [15,16].

Detection of the pathovars causing BB in field pea was confirmed as early as 3–4 days after infection which was one week earlier than the characteristic visual BB symptoms appeared [3,20]. Early detection of BB in the field can help in studying host-pathogen interactions for the two pathovars and phenotyping resistance in field pea breeding trials. These LAMP assays also accurately detected the presence of Psp or Pss in seed samples, further broadening their potential use.

Both assays achieved 100% specificity on our validation panel of 95 bacterial isolates. No false positives or false negatives were observed when the assays were validated with the alternate field pea pathovar, 37 isolates each of Psp and Pss and 18 strains of *Pseudomonas* species and other bacteria. The results for the two LAMP assays also correlated with established lab-based culturing and PCR methodology.

Ten isolates that represented Psp races 2, 3, 4, 6, and 7 were incorporated into this study to ensure the genetic diversity within the target group would be detected by the Psp LAMP assay. This is particularly important for *Pseudomonads*, which are a biologically and genetically diverse group [30]. Races 2, 3, and 6 are present in Australia and Race 4, which is present in the UK, was also included. The Psp LAMP assay identified isolates of these races correctly indicating that the LAMP assay can be applied in other parts of the world.

For Pss, the *salA* gene was a good target for primer design as it is present in all Pss representative genomes available in NCBI and Pss used in this study. The *salA* gene is a key regulatory element for syringomycin production which is essential for virulence, growth, and survival for Pss [31]. The *salA* gene functions as a transcriptional activator of more than one gene or operon for three major toxins syringomycin, syringopeptin and syringolin responsible for virulence effects in Pss [27]. The LAMP assay for Pss was validated on isolates that were collected from various hosts including field pea and bean as well as horticulture crops such as Orange (*Citrus sinensis*), Plum (*Prunus domestica*) and Impatiens species, showing the potential capability of the LAMP assay for Pss surveillance activities in other crops (although this has not been validated). In this study, the Pss LAMP assay was able to differentiate Pss, *Pseudomonas viridiflava* and *Pseudomonas cichorii*, all species that can infect a broad range of plant species and cause blight diseases [32,33].

Testing of naturally infected field pea tissue and seed samples from Victorian surveillance dating from 1983 to 2019 with the Psp and Pss-specific LAMP assays only detected the presence of Pss. This result was confirmed using PCR. It was reported previously for the first time by Richardson et al., (2011) that reports from the 1990's showed Psp as the most devastating of the two pathovars in Australia [7,9]. LAMP assays detected Pss, which were reconfirmed with PCR shows that these LAMP assays can be utilized for the large sample testing efficiently and to provide clear knowledge of the pathovar prevalence in field pea pre-breeding research, breeding trials, and farmers crops.

In summary, two LAMP assays were developed that have been successfully shown to identify *Pseudomonas syringae* infecting field pea to the pathovar level, as Psp or Pss. These assays offer portability to conduct tests in the laboratory or in field. The assays have rapid time to positive outputs, within 20 min, which is significantly quicker than the conventional PCR methods available for pathovar specificity. The assays were suitable for testing naturally infected plant tissue and seed samples for the presence of Psp and Pss without isolating the bacteria. The assays were shown to be rapid and sensitive, being capable of detecting their target pathogens *in-planta* before visual symptoms were observed. The suitability of these tests for surveillance is further evident by the assays already being taken up by BB field pea surveillance that informs the Australian breeding program.

Author Contributions: Conceptualization, P.K., G.R., B.R.; methodology, P.K., R.M.; bioinformatics, P.K., M.F.; experiments, P.K.; writing and review P.K., B.R., R.M., G.H., G.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received joined funding from Grains Research and Development Corporation (GRDC) and Agriculture Victoria under project “DAV1607-010BLX 9176093 PulseBio 3 Stable grain yield in pulses through improved stress tolerance”.

Conflicts of Interest: The authors declare no conflict of interest.

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