Development of a TaqMan PCR assay for specific detection and quantification of *Pectobacterium brasiliense* in potato tubers and soil

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Abstract

Pectobacterium brasiliense (Pb) is one of the causal agents of soft rot and blackleg diseases and has become a pathogen of economic importance in many potato production regions worldwide. Accurate, sensitive and timely identification of Pb is critical for improved management of the pathogen to mitigate yield losses. This study describes the development and validation of a TaqMan probe-based quantitative real-time PCR assay for rapid and specific detection of Pb in plant material and soil. A primer-pair that amplifies a 125-bp fragment was designed from the 16S-23S intergenic spacer region ribosomal RNA and the tRNA-Glu gene region. The specificity of the assay was evaluated with 24 isolates representative of nine different *Pectobacterium* and *Dickeya* species associated with soft rot and blackleg of potatoes. The designed Pb species-specific primers and FAM-labelled TaqMan probe were specific for detection of Pb in all the assays performed and it did not detect other *Pectobacterium* and *Dickeya* species. The TaqMan PCR assay could detect Pb DNA quantities as low as 10 fg/µl and DNA from a concentration of cells as low as 10^3 colony forming units/ml. The assay was capable of identifying and quantifying Pb in potato tubers and in field soils without the interference of inhibitors. The developed TaqMan PCR assay can be used for routine Pb diagnostics, surveillance and epidemiological studies.

Keywords: Pectobacteriaceae, seed tuber, soft rot, latent infection, qPCR

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Introduction

Pectobacterium and *Dickeya* species are economically important pathogens of potato as well as other vegetable crops worldwide (Czajkowski et al. 2011; Sławiak et al. 2013). *Pectobacterium* species produce pectinolytic and cellulolytic enzymes that degrade cell walls resulting in infiltration and maceration of plant organs and tissues causing diseases like blackleg, stem rot and tuber soft rot on potatoes (Czajkowski et al. 2011; Kettani-Halabi et al. 2013; Lee et al. 2014). Of importance is blackleg and tuber soft rot that cause extreme yield losses to potato production in the field, during transit and in storage (Czajkowski et al. 2011).

Pectobacterium is a complex taxon consisting of strains with a range of hosts, different phenotypic, biochemical and genetic characteristics (Kettani-Halabi et al. 2013). Previously classified under the genus *Erwinia*, the extensive taxonomic revision led to recognition of the genus *Pectobacterium* (Hauben et al. 1998). Phylogenetically informative genes further provided consistent support for separating the *Pectobacterium* genus into *Pectobacterium* and *Dickeya* (Brady et al. 2012; Nabhan et al. 2012; Adeolu et al. 2016). To date, the *Pectobacterium* genus includes numerous formally described species, that is, *P. carotovorum, P. zantedeschiae, P. atrosepticum, P. parmentieri, P. wasabiae, P. punjabense, P. betavasculorum, P. aroidearum, P. peruviense, P. polaris* and *P. aquaticum* (Czajkowski et al. 2015; Zhang et al. 2016; Dees et al. 2017; Waleron et al. 2017; Sarfraz et al. 2018; Walerona et al. 2019), and Candidatus *P. maceratum* (Shirshikov et al. 2018). *Pectobacterium carotovorum* species was previously subdivided into four subspecies, namely *carotovorum*, *brasiliense*, *actinidiae* and *odoriferum* (Duarte et al. 2004; Waleron et al. 2019). Recent revisions of this taxonomy, however, have elevated *P. carotovorum* subsp. *actinidiae*, *P. carotovorum* subsp. *brasiliense* and *P.*

carotovorum subsp. *odoriferum* to species level, as *Pectobacterium actinidiae* sp. nov., *Pectobacterium brasiliense* sp. nov., and *Pectobacterium odoriferum* sp. nov., respectively (Portier et al. 2019).

Of the *Pectobacterium* species, a number of reports have shown that *P. brasiliense* (Pb) can cause high yield losses in potato production (McNally at al. 2017; van der Wolf et al. 2017). Initially reported in Brazil (Duarte et al. 2004), Pb has emerged as a pathogen of economic importance in many potato producing countries (van der Merwe et al. 2010; De Boer et al. 2012; Ngadze et al. 2012; Panda et al. 2012; Leite et al. 2014; Onkendi et al. 2014). In South Africa, there has been an increase in outbreaks and severity of blackleg and soft rot diseases (van der Merwe et al. 2010; Ngadze et al 2012). This is possibly due to warmer prevailing temperatures favouring Pb and an increase in cultivation of susceptible varieties.

Managing *Pectobacterium* species is challenging as the pathogens are widespread and can survive latently in seed tubers (De Boer et al. 2012). Disease control under field conditions based on physical, chemical and biological methods has failed (Czajkowski et al. 2011). Attempts to breed for resistance have not been successful (Marquez-Villavicencio et al. 2011). The current practical approach for controlling Pb in potato fields is based on planting pathogen-free potato seed materials in uninfested potato fields (Czajkowski et al. 2009). Therefore, it is imperative to develop accurate, rapid and robust diagnostic tools to support seed certification programmes, and implement prophylactic disease management strategies that can prevent yield losses caused by the pathogen.

In the genus *Pectobacterium*, species have traditionally been identified according to their morphology and host range (Toth et al. 1996). Unfortunately, morphological characteristics and symptom expression on the same host overlap between species (Waleron et al. 2017). Identification using morphology therefore requires trained personnel to consistently distinguish species. Furthermore, these traditional methods have poor reproducibility, are time-consuming and labour intensive. Cost- and time-sensitive decisions are made when implementing disease control strategies like crop destruction, seed certification and quarantine programs, so the tests used must be rapid, sensitive and specific (Czajkowski et al. 2015).

Alternative pathogen detection methods have focused on serological and nucleic-acid based assays (Ward et al. 2004). The most widely used methods for detection of plant pathogens include Enzyme Linked Immunosorbent Assay (ELISA) (Tsror (Lahkim) et al. 2012) and Polymerase Chain Reaction (PCR) (Kang et al. 2003; Potrykus et al. 2014). Isothermal detection methods such as recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP) (Li et al. 2011; Ahmed at al. 2018) have also been used. Of these, PCR and other DNA-based methods are preferred for their sensitivity, specificity, and the ease with which the required reagents can be obtained. Duarte

et al. (2004) developed species-specific primers for identification of Pb. Potrykus et al. (2014) designed a conventional multiplex PCR technique for identification of *P. atrosepticum, P. carotovorum* subsp. *carotovorum, P. wasabiae* (isolates later reclassified as *P. parmentieri),* together with *Dickeya* spp. The inherent problem of conventional PCR assays is their low sensitivity and requires post-PCR handling to visualize the results for pathogen identification; as such they are prone to errors. Real-time polymerase chain reaction (qPCR) has been extensively applied in detection and quantification of numerous pathogens from plant material (Ammour et al. 2019; Rahimi-Khameneh et al. 2019). Kim et al. (2012) developed a SYBR Green qPCR assay for detection of *P. parmentieri* based on the YD repeat protein gene. Pritchard et al. (2013) developed a TaqMan PCR (qPCR) assay based on an unidentified gene region for identification of *P. atrosepticum.* Van der Wolf et al. (2017) developed a TaqMan PCR assay for identification of Pb based on the sequences of the transcriptional regulator *araC* gene region. This is currently the only publicly available TaqMan PCR assay for the detection and quantification of Pb, but this test has not been evaluated for potential detection of Pb in soil. Therefore, there is a need for an additional rapid, sensitive, specific and robust Pb diagnostic test that will complement existing TaqMan PCR assays in detection and quantification of Pb in various matrices including soil and seed tubers, to facilitate effective disease management decisions. An additional diagnostic test based on a different gene region will be invaluable in confirmation testing and troubleshooting in diagnostic laboratories.

The primary objective of this study was therefore to develop and validate a highly specific and sensitive TaqMan PCR assay (referred to as JW2020 in this manuscript) for the detection and quantification of Pb. The second objective was to demonstrate potential applications of the TaqMan PCR assay in testing potato tubers and soil samples for the presence of Pb.

Materials and methods

Bacterial strains

Bacterial strains used in this study are shown in Table 1. The identity of the *Pectobacterium* isolates was confirmed using previously designed species-specific primers by conventional PCR amplification (Duarte et al. 2004; Potrykus et al. 2014). The Pb strain JJ68 used for validation was obtained from the University of Pretoria culture collection. The isolate was grown at 28ºC for 48 h on crystal violet pectate medium (CVP) (Hyman et al. 2001) and maintained on nutrient agar (NA) and nutrient broth prior to DNA extraction. DNA was extracted using the ZR soil microbe DNA mini Prep™ kit (Zymo Research, California, USA) according to the protocol provided by the manufacturer and stored at -20ºC for downstream analyses.

Table 1 *Pectobacterium* and *Dickeya* species used for specificity testing of the *Pectobacterium brasiliense* species-specific primers in TaqMan PCR JW2020

Species	Isolate number	Geographic origin	Host	TaqMan PCR test[a]
Pp	IPO 1949	Netherlands	*Solanum tuberosum*	-
Pp	IPO 1952	Netherlands	*Solanum tuberosum*	-
Pp	IPO 1953	Netherlands	*Solanum tuberosum*	-
Pp	IPO 1954	Netherlands	*Solanum tuberosum*	-
Pcc	IPO 1961	Netherlands	*Solanum tuberosum*	-
Pcc	PPO 9053	Netherlands	*Iris* sp.	-
Pcc	PPO 9116	Netherlands	*Solanum tuberosum*	-
Pcc	PPO 9181	Netherlands	*Zantedeschia* sp.	-
Pcc	PPO 9180	Netherlands	*Zantedeschia* sp.	-
Pcc	PPO 9054	Netherlands	*Iris* sp.	-
Pcc	PPO 9055	Netherlands	*Hyacinthus* sp.	-
Pcc	PPO 9085	Netherlands	*Zantedeschia* sp.	-
Pcc	PPO 9094	Netherlands	*Hyacinthus* sp.	-
Pcc	PPO 9123	Netherlands	*Solanum tuberosum*	-
Pcc	91/84	Switzerland	*Solanum tuberosum*	-
Pb	PRI 3644	Netherlands	*Solanum tuberosum*	+
Pb	PRI 3649	Netherlands	*Solanum tuberosum*	+
Pb	PRI 3666	Netherlands	*Solanum tuberosum*	+
Pb	PRI 3669	Netherlands	*Solanum tuberosum*	+
Pb	PRI 3708	Netherlands	*Solanum tuberosum*	+
Pb	PRI 3709	Netherlands	*Solanum tuberosum*	+
Pb	PRI 3710	Netherlands	*Solanum tuberosum*	+
Pb	PRI 3711	Netherlands	*Solanum tuberosum*	+

Pb	JJ 147	South Africa	*Solanum tuberosum*	+
Pb	JJ68	South Africa	*Solanum tuberosum*	+
Pb	LMG 21371	Brazil	*Solanum tuberosum*	+
Pa	1371	Netherlands	*Solanum tuberosum*	-
Pa	1066	Netherlands	*Solanum tuberosum*	-
Pa	1071	Netherlands	*Solanum tuberosum*	-
D. zeae	2132/CFBP4176	UK	*Chrysanthemum morifolium*	-
D. solani	2222	Netherlands	*Solanum tuberosum*	-
D. dadantii	2122	Cuba	*Ipomea batatas*	-
D. dieffenbachiae	2124/CFBP1360	France	*Dieffenbachia* sp.	-
D. chrysanthemi	2117/CFBP1236	USA	*Parthenium argentatum*	-
D. paradisiaca	2127/CFBP3477	Colombia	*Musa paradisiaca*	-

[a]TaqMan PCR test results: + sign indicates amplification, - sign indicates no amplification

Pp - *Pectobacterium parmentieri*, Pcc - *Pectobacterium carotovorum* subsp. *carotovorum*, Pb - *Pectobacterium brasiliense*, Pa - *Pectobacterium atrosepticum*

TaqMan PCR assay design and specificity

To develop a TaqMan PCR assay, sequences for the target Pb and related species and subspecies were obtained from NCBI's GenBank (Fig. 1). The DNA sequences of 16S-23S internal transcribed spacer (16S-23S ITS) and tRNA-Glu gene region which contain polymorphisms informative for the discrimination of closely related *Pectobacterium* species were aligned using ClustalW in MEGA 5 (Tamura et al. 2011). Primer Express Software version 2.0 (Thermo Fisher Scientific, Massachusetts, United States) was used to design a unique set of Pb species-specific TaqMan® primers Pb1F/Pb2R (Table 2) with an amplification product of 125 bp and a dual labelled fluorogenic probe PbPr based on regions of the greatest sequence dissimilarity among species. The probe (PbPr) was modified at the 5′-end with the fluorescent reporter dye 6-FAM (6-carboxy-fluoroscein), an internal ZEN Quencher, and a Black Hole Quencher 1 (BFQ 1), at the 3' end. The primers and a probe were designed for optimum specificity by including as many mismatches with closely related species.

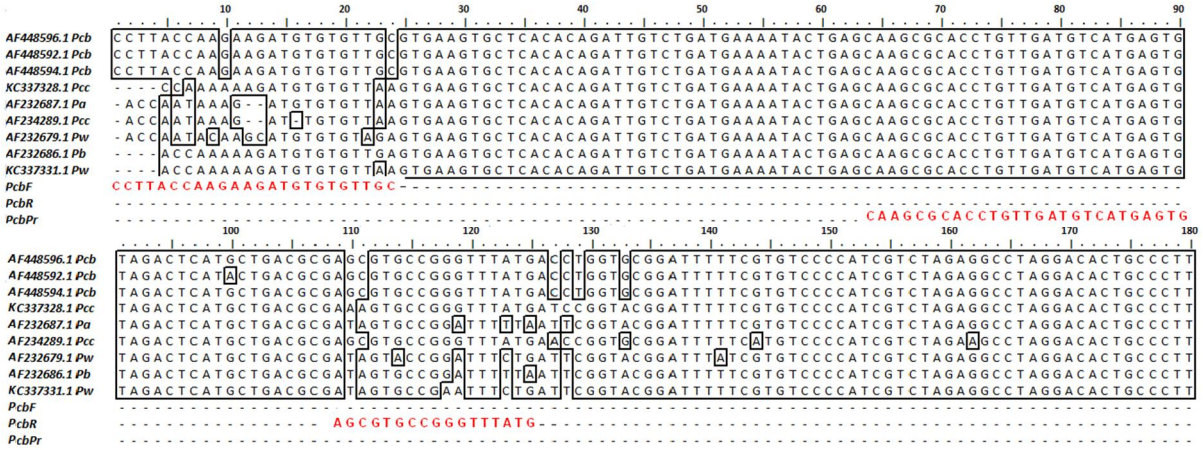

Fig. 1. Alignment showing positions of the designed *Pectobacterium brasiliense* primers and probes on the 16S-23S intergenic spacer region ribosomal RNA and tRNA-Glu nucleotide sequence in comparison to other *Pectobacterium* species. Coloured bases are the primer and probe.

The designed primers and probes were assessed for specificity to Pb and other *Pectobacterium* spp. prior to synthesis using the Primer-BLAST algorithm in NCBI GeneBank nucleotide data base (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). This allowed comparison of *Pectobacterium* spp. that were not included for qPCR testing in this study. The melting temperature, potential formation of secondary structures, and interactions among the oligonucleotide sequences were also evaluated *in silico* using the Primer-BLAST software (Ye et al. 2012). Primers and probes (Table 2) were synthesised by Integrated DNA Technologies Inc. (Iowa, USA) and evaluated by TaqMan PCR using DNA extracted from a panel of phylogenetically related *Pectobacterium* and *Dickeya* strains (Table 1). In addition, the inclusivity of the assay was evaluated by testing it with DNA originating from 11 Pb isolates originating from four different countries. This was performed in a PikoReal 96 thermal cycler

(Thermo Fisher Scientific, California, United States) within 96 well plates sealed with a transparent adhesive seal.

Table 2. TaqMan PCR diagnostic primer and probe sets used for detection and quantification of *Pectobacterium brasiliense*

Primer / probe name	Description	Sequence (5'-3')	Tm (°C)	GC (%)	Reference
Pb1F	Forward	CCTTACCAAGAAGATGTGTGTTGC	61	46	This study JW2020
Pb2R	Reverse	CATAAACCCGGCACGCT	59	59	
PbPr	FAM-BHQ1 labelled probe	CAAGCGCACCTGTTGATGTCATGAGTG	63	52	
PbrFw	Forward	TGCGGGTTCTGCGTTTC	60	59	van der Wolf et al. (2017)
PbrRv	Reverse	TGGCGCGTTCGCAATAT	59	53	
Pbrb	FAM-MGB labelled probe	CAAGGCACGATACG	48	57	

Tm = Melting temperature; GC = Percentage of guanine and cytosine

The TaqMan PCR assay was performed in a 10 µl volume consisting of 5 µl of TaqMan Universal Master Mix II (Applied Biosystems, California, United States), primers (0.3 µM) and probe (0.1 µM), 2 µl of DNA, and PCR water added to a final volume of 10 µl. Thermal cycling conditions consisted of 50ºC for 2 min, 95ºC for 10 min, 45 cycles of 95ºC for 15 s and 60ºC for 1 min. No-template control (NTC) reactions were included in which the DNA was substituted with molecular biology grade water (Sigma-Aldrich, Missouri, United States). For quantification purposes, a standard curve was prepared using serially diluted DNA of known concentration (10 ng/µl to 1 fg/µl) extracted from Pb reference isolate JJ68. Three replicates from each dilution were included in each PCR run. The PikoReal 96 thermocycler software was then used to determine DNA concentration of each sample. The standard curve was also used to determine PCR amplification efficiency (E), calculated using the equation $E = (10^{-slope} - 1) \times 100$.

Assay sensitivity

The analytical sensitivity of the TaqMan PCR assay was evaluated using DNA originating from a pure culture of Pb isolate JJ68 that was grown overnight in nutrient broth at 37ºC in a shaking incubator (Stuart Orbital SI500, UK). Cells were suspended in Ringer's solution to an optical density of 0.1= 10^8 CFU/ ml (600 nm) and DNA extracted as previously described. Purified genomic DNA was quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, California, United States) with the Qubit dsDNA HS Assay kit according to the manufacturer's instructions (Thermo Fisher Scientific, California, United States). DNA was then serially diluted in sterile water to determine the sensitivity (limit of detection).

The assay developed in this study was compared with the TaqMan assay developed by van der Wolf et al (2017) by evaluating the variability of the Ct values obtained after amplification of eight serial dilutions (10^9 to 10^2 CFU/ml) of the Pb isolates Pb3649 (Netherlands) and JJ147 (South Africa). Genomic DNA was extracted from each dilution and qPCR carried out as previously described except that a different set of primers and probes designed by van der Wolf et al. (2017) were used for another run. The Ct values were plotted against the bacterial concentration (CFU/ml). Furthermore, repeatability was analysed among triplicates of an assay (within-run), whereas reproducibility was determined in three repeated day assays (between-runs) by evaluating the variability of the Ct values obtained after amplification of eight-fold dilutions (10^9 to 10^2 CFU/ml) of the Pb isolate LMG21371. The coefficient of variation (CV) was determined for each of the dilutions by dividing the standard deviation (SD) with the mean of Ct values.

Detection of Pb in potato tubers

To evaluate the ability of the TaqMan PCR assay to detect and quantify Pb in plant material, mini-tubers (cv. Mondial) were surface sterilized with 10% sodium hypochlorite for 1 min and washed three times with sterilized water. Mini-tubers were then cut into thin slices using a sterile scalpel blade. Cut potato slices were placed inside Petri dishes containing filter paper with 2 ml of sterile water to create moisture chambers. Bacterial colonies (10^8 CFU/ml) of Pb isolates (JJ68, JJ83, JJ147), Pp isolates (G620), Pcc isolates (Bcc396) were stab inoculated into the middle of potato slices using sterile toothpicks and incubated for 18 h at 28°C. Tuber slices stabbed with sterile water alone served as negative controls. There were three replicates for each isolate. Afterwards, the tuber slices were pulverised in Bioreba bags and DNA was extracted from tuber tissue using ZR soil microbe DNA mini Prep™ kit (Zymo Research) according to the protocol provided by the manufacturer.

The TaqMan PCR assay was also used to test 276 DNA samples of potato tubers in 2013, 2014 and 2015 at the Dutch General Inspection Service for Agricultural Seed and Seed potatoes (NAK). The heel-end of each potato tuber was carefully removed using a sterile scalpel blade, macerated and the extracts enriched in Pectate Enrichment Broth for 72 h at 25°C to detect possible latent infections. After enrichment DNA was extracted using the Agowa sbeadex maxi plant kit (LGC, Teddington, UK) in conjunction with a KingFisher Flex Magnetic Particle Processor (Thermo Fisher Scientific, California, United States) according to manufacturer's instructions.

Detection of Pb in soil

The utility and sensitivity of the assay was evaluated by detection and quantification of Pb in the soil. Field soils were collected and placed in 2 l greenhouse pots. The soils were not autoclaved or pasteurised in order to maintain the biological, physical and chemical integrity. The Pb JJ68 isolate at a concentration of 10^8 CFU/ml Ringer's solution was added to the soil in varying amounts to obtain final concentrations of 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 CFU/g soil. Soil amended with Ringer's solution alone served as negative control. The inoculated soils were homogenized and incubated for 12 h at 25^{o}C. A total of 10 g of soil was taken from each sample and homogenised in a sterile 50 ml Falcon tube before 0.25 g was subsampled for DNA extraction. DNA was extracted in triplicate using the ZR soil-microbe DNA mini Prep™ kit (Zymo Research) according to the manufacturer's protocol. DNA was amplified and quantified using TaqMan PCR as described above.

Results

Primer design

Multiple sequence alignment of the complete 16S-23S ITS and tRNA-Glu gene region identified nucleotide polymorphisms unique to Pb, and in appropriate positions for primer design (Fig. 1). Primer-BLAST of the Pb primer sequences Pb1F and Pb2R reported a 125-bp product from the 16S-23S intergenic spacer region ribosomal RNA and tRNA-Glu gene region (GenBank Acc. No. AF448596.1), matched with only Pb 16S-23S ITS and tRNA-Glu sequences (CP020350.1, CP009769.1, AF448596.1, AF448594.1). No significant primer or probe BLAST hits were recorded with any other *Pectobacterium* species or its close relatives such as *Dickeya* or other known bacterial and fungal pathogens of potato. The specificity of TaqMan PCR probes and primers was also verified *in silico* through multiple sequence alignment and were found to be highly specific to Pb.

Specificity of the assay

The specificity of the designed primers (Pb1F/Pb2R) and probe (PbPr) was evaluated using soft rot enterobacterial pathogens, which are phylogenetically related or have potential to be found in the same ecological niche with Pb. This included 11 Pb strains, 11 *P. carotovorum* subsp. *carotovorum*, three *P. atrosepticum*, four *P. parmentieri* and six different *Dickeya* spp. strains (Table 1). The TaqMan assay only detected Pb strains, no amplification was observed in DNA originating from non-Pb species (Table 1).

TaqMan PCR assay sensitivity

The analytical sensitivity of the assay was determined by using pure genomic DNA from Pb isolate JJ68. Figure 2 shows the standard curve of the eight serial dilutions of Pb DNA assayed using the TaqMan PCR assay. A high negative correlation ($r^2 = 0.9991$) was observed between the Ct values and purified Pb DNA (Fig. 2), slope of -3.65 and a reaction efficiency of 103.9% calculated in the linear zone according to the MIQE guidelines (Bustin et al. 2009). The assay displayed high sensitivity, with a detection limit of 10 fg/µl Pb genomic DNA, with Ct < 40.

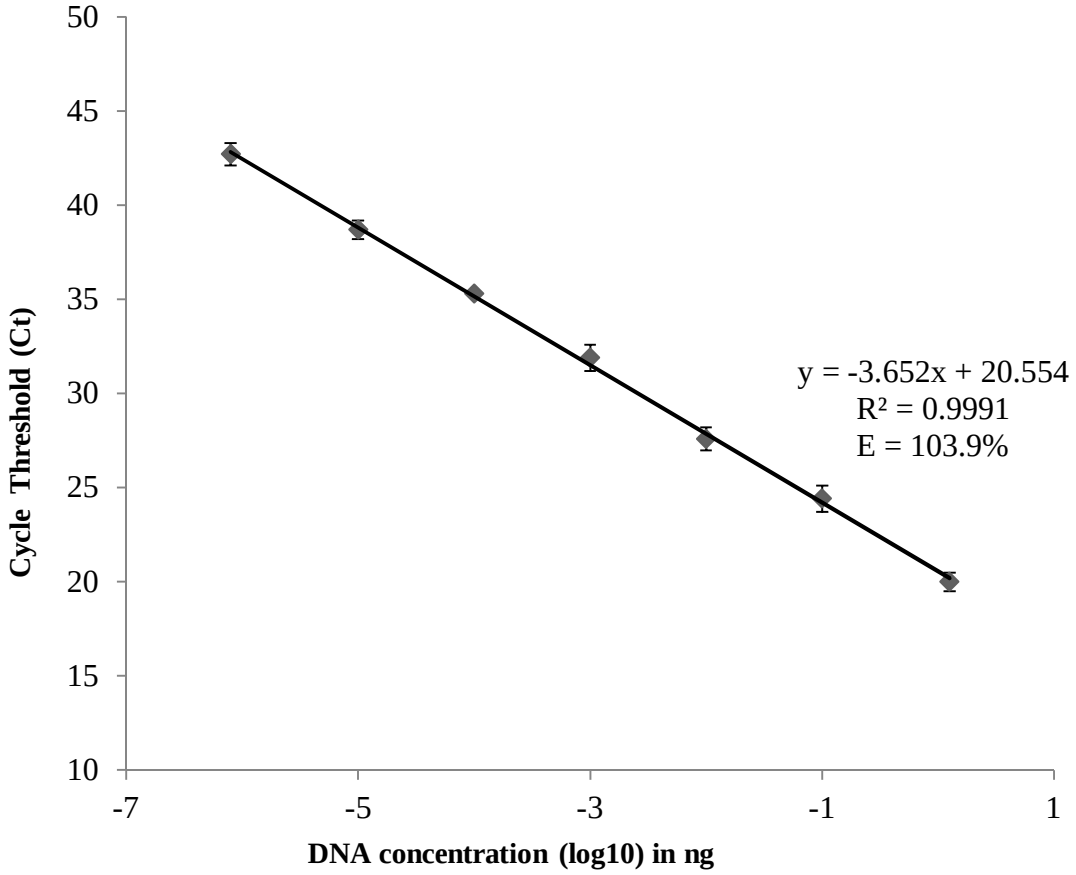

Fig. 2. DNA concentration verse cycle threshold with reaction efficiency, generated by TaqMan PCR using Pb1F and Pb2R primers and PbPr probe. The regression equation, the coefficient of determination (R^2) and reaction efficiency (E) are indicated. Each point represents the mean of three replicates. Bars represent the standard deviation of the mean.

The reliability and performance of the TaqMan PCR assay developed in this study were evaluated by comparing it with the TaqMan PCR assay developed by van der Wolf et al. (2017). The Ct values of eight serial dilutions (10^9 to 10^2 CFU/ml) of two Pb isolates, Pb3649 (Netherlands) and JJ147 (South Africa), showed that the R^2 of each assay and all the isolates was greater than 0.99 (Figs. 3A and 3B).

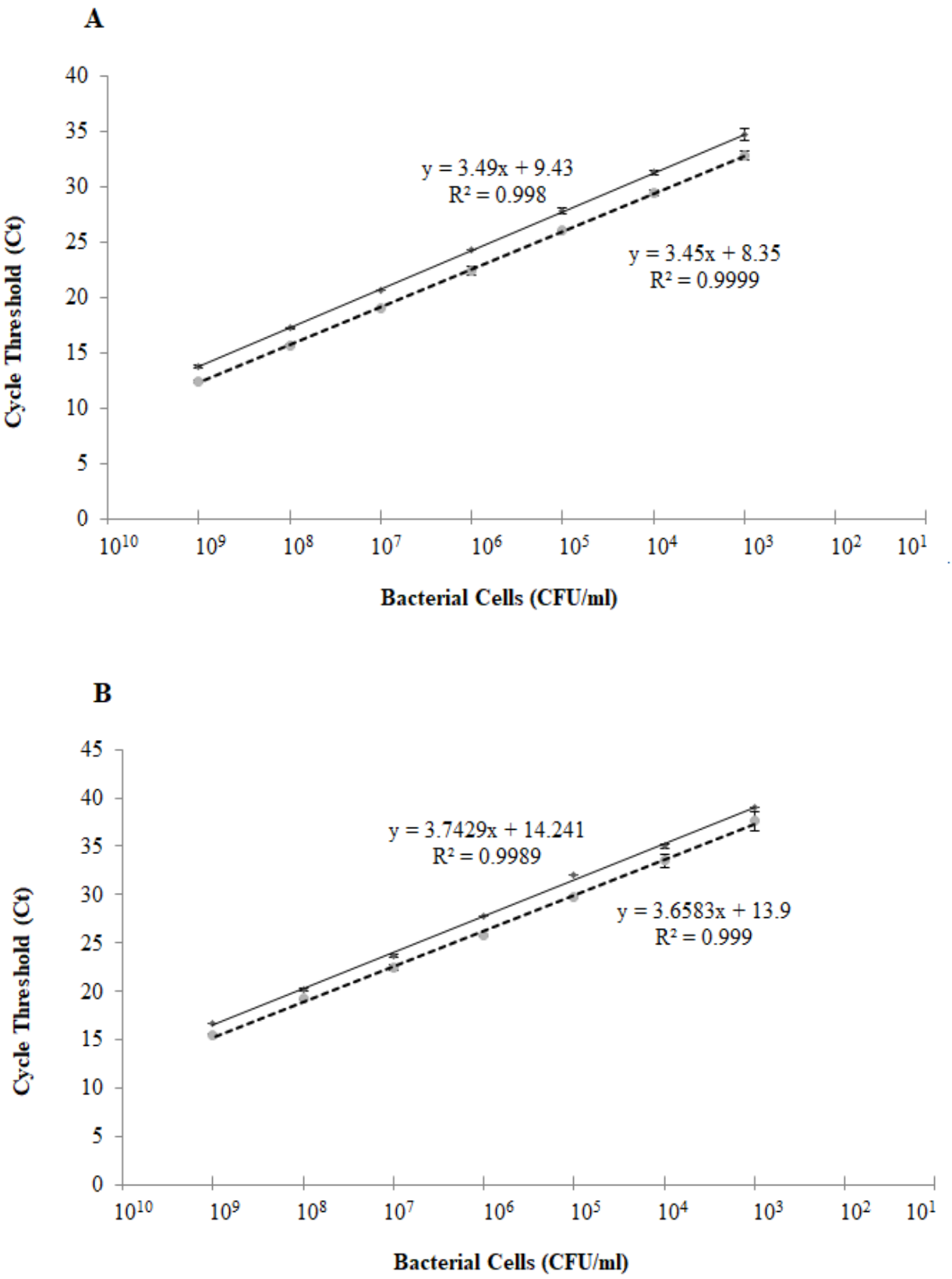

Fig. 3. Standard curves from TaqMan PCR assay (JW2020) designed in this study, shown by a dashed line (--), and a TaqMan assay developed by van der Wolf et al. (2017) shown by a solid line (-). Ct values of DNA extracted from eight-fold serial dilution of Pb isolates A) LMG 3649 and B) JJ147, were plotted against the concentration of bacterial cells. Error bars represent standard deviations.

The repeatability and reproducibility of the TaqMan PCR assay were evaluated by analysing the variability of Ct values of eight serial dilutions (10^9 to 10^2 cfu/ml) of isolate LMG 21731. The CV of the mean Ct values obtained for the genomic DNA standard curve ranged from 0 to 2% and 0.5 to 4.5% for intra-assay and inter-assay respectively indicating the high reproducibility of the assay (Table 3).

Table 3. Variability of Ct values within a single run (intra laboratory assay) and between different runs (inter-laboratory assay) for the *Pectobacterium brasiliense* TaqMan PCR assay

Intra-assay variability			**Inter-assay variability**	
Concentration	**Mean Ct±SD**	**CV[a] (%)**	**Mean Ct±SD[b]**	**CV (%)**
1E+9	13.8	0	13±0.15	1.1
1E+8	17.25±0.25	1.4	16.5±0.1	0.6
1E+7	20.7	0	19.9±0.2	1.0
1E+6	24.3	0	23.35±1.05	4.5
1E+5	27.73±0.2	0.7	26.8±0.5	1.8
1E+4	31.25±0.3	0.9	30.4±075	2.46
1E+3	34.9±0.9	2.0	33.85±0.2	0.5
1E+2	35.9±4.1	0.11	37.8±0.8	2.1

[a]Coefficient of variation

[b]Standard deviation

Detection of Pb in plant samples

Assay specificity and sensitivity was further evaluated with DNA extracted from potato tuber material inoculated with Pb, Pcc and Pp. A Ct value of 30 or less was observed when DNA originating from potato tuber slices inoculated with Pb were tested with the assay. No detection was observed with DNA originating from tuber material inoculated with Pcc, Pp or non-inoculated control material. The TaqMan PCR assay was also used to detect the presence of Pb in DNA extracted from asymptomatic potato tubers from the Netherlands (Table 4). A total of 276 DNA samples were analysed by NAK, 151 samples were positive with the TaqMan PCR assay developed in this study, while 145 samples tested positive for Pb using the TaqMan PCR assay developed by van der Wolf et al. (2017).

Table 4. Comparison of two TaqMan PCR assays for the detection of *Pectobacterium brasiliense* in potato tubers

		Van der Wolf Pb TaqMan assay[a] Pb		Pb TaqMan assay JW2020Pb	
Year DNA	**N**	**Positive samples**	**Mean Ct**	**Positive samples**	**Mean Ct**
2013	92	43	24.9 (0.2)	43	24.3
2014	92	51	19.9	54	21.5
2015	92	51	22	54	21
Total	276	145	22.3	151	22.3

[a]Assay developed by van der Wolf et al. (2017)

Detection of Pb in the soil

The amount of Pb in field contaminated soil was quantified using the TaqMan PCR assay developed in this study. The Ct values of positive samples ranged from 9 to 34, corresponding to Pb DNA concentration of 4 ng to 40 fg DNA/g of soil (Fig. 4).

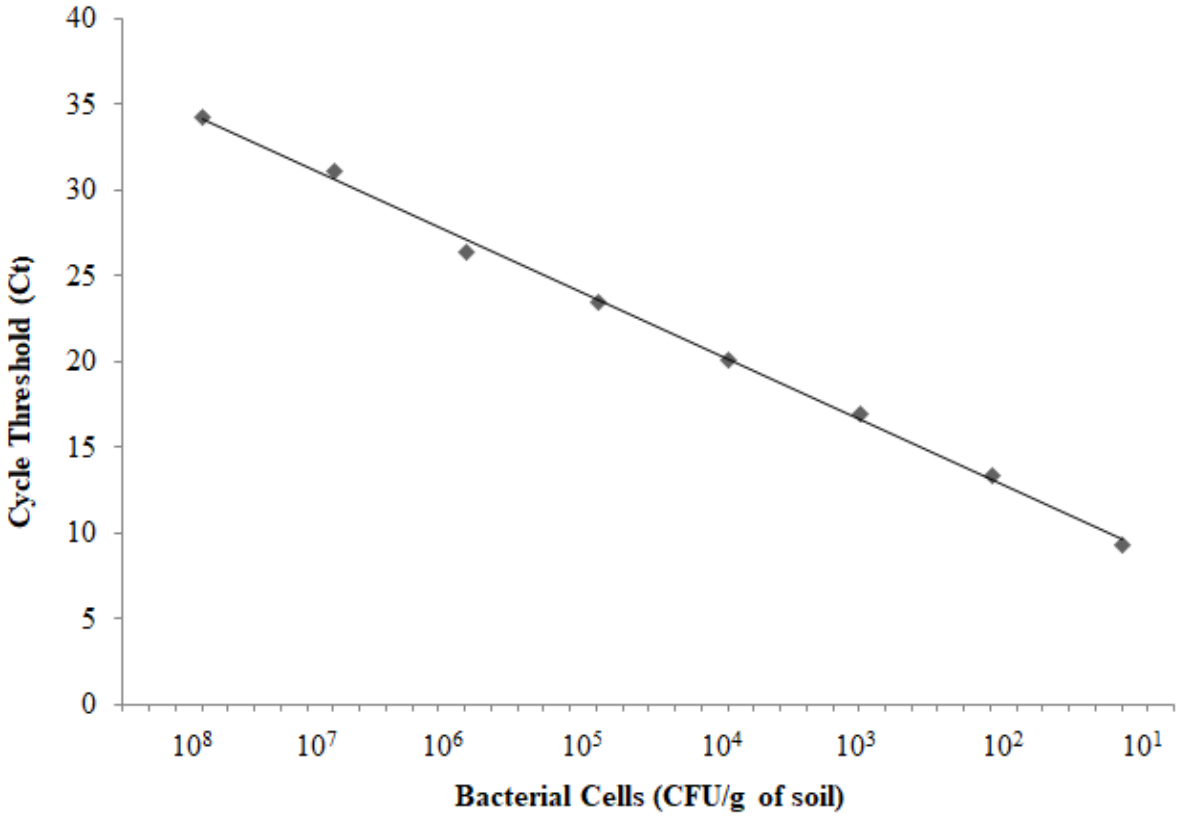

Fig. 4 Standard curve for quantification using the TaqMan PCR assay of Pb DNA from soil artificially infested with *Pectobacterium brasiliense*.

Discussion

This study describes the development and validation of a new TaqMan PCR assay for sensitive and specific detection of Pb in potato material and soil. The TaqMan PCR protocol was designed using a pair of primers and a TaqMan probe targeting the16S-23S rDNA ITS and tRNA-Glu gene regions. The 16S-23 ITS and tRNA-Glu gene regions have high nucleotide variability for differentiating Pb from other *Pectobacterium* species.

Pectobacterium brasiliense has traditionally been identified using classical morphological methods, which are ambiguous since *Pectobacterium* species can co-occur in the same symptomatic tissue, making it difficult to differentiate among closely related species (Czajkowski et al. 2015). Conventional PCR methods for identification and discrimination of *Pectobacterium* species have been developed (Duarte et al. 2004; Potrykus et al. 2014) but do not allow for quantification of pathogens. There is a general consensus that qPCR is faster, more sensitive, and more accurate for detection and quantification of plant pathogens than conventional PCR and traditional techniques (Narayanasamy 2011; Czajkowski et al. 2015; Martinelli et al. 2015). Real-time PCR assays targeting the detection and quantification of *Pectobacterium* species have been developed for *P. parmentieri* (Kim et al. 2011), *P. atrosepticum* (Pritchard et al. 2013) and *P. brasiliense* (van der Wolf et al. 2017). Similarly, in the present study we developed a TaqMan PCR assay for detection and quantification of Pb in plant material and soil.

Significant high sensitivity and specificity are the important attributes of a good TaqMan PCR assay. The diagnostic method developed in this study is sufficiently sensitive and highly specific to detect DNA extracted from pure cultures of Pb, potato tubers infected with Pb, and soil matrices. It was able to discriminate Pb from other phylogenetically related *Pectobacterium* species that are known to co-exist with Pb. Furthermore, the assay was able to quantify the pathogen in plant and soil samples. The assay showed positive results for Pb only while all the other related species tested negative.

In addition to being highly specific, the assay was also shown to be extremely sensitive. Diagnostic methods should be sufficiently sensitive to detect a low amount of the target pathogen in an infected sample without producing false negative results. The standard curves generated by the TaqMan PCR in seven-fold dilutions of Pb demonstrated good coefficients of correlation for the PCR primers and TaqMan probe used. The limit of detection of the TaqMan PCR assay corresponded to 10^3 CFU/ml and 20 fg of genomic DNA. Similar results were found by Kim et al. (2012) and van der Wolf et al. (2017). A similar detection limit using qPCR has been reported for other bacterial pathogens (Weller et al. 2000; Kelly et al. 2012).

The reliability of the TaqMan PCR assay developed in this study was confirmed when it performed comparably well with that developed by van der Wolf et al. (2017). Van der Wolf et al. (2017) found Ct < 35 as positive, while the TaqMan developed in this study had a Ct < 40. As stated by Grosdidier et al. (2017), late Ct values can be reliable if a consistent cut-off value has been determined, as in the case here. The coefficient of variation ranged from 0.1 to 4.5%, an indication that the developed TaqMan qPCR assay can produce consistent, reproducible and reliable results. When tested against 276 stored DNA samples (2013-2015) extracted from asymptomatic tubers the assay was able to positively detect Pb in more samples than any of the available Pb assays indicating that the assay is accurate, robust and specific for detection of Pb.

Currently, the most effective method to manage *Pectobacterium* species is planting pathogen-free seed tubers in uncontaminated soil (Czajkowski et al. 2011). Although widely accepted that planting pathogen-free seed is key in disease management, no country makes use of official post-harvest diagnostic tests for the Soft Rotting Pectobacteriaceae (de Werra et al. 2020). *Pectobacterium brasiliense* can survive in infected plants and debris in the soil for up to four weeks and is spread through asymptomatic seed tubers, irrigation water and insect vectors (van der Wolf et al. 2017). The developed TaqMan qPCR assay could therefore be used for the detection and quantification of Pb in diverse sample types such as irrigation water and seed tubers to avoid introduction of inoculum into clean fields. Most importantly, this assay is suitable for use as a rapid diagnostic tool in seed certification systems as it reduces the testing time compared to other diagnostic methods and can also detect Pb in latently infected tubers. This provides a first step towards management of Pb in the potato industry as contaminated seed is the main means of transmission of the bacteria.

In conclusion, the developed TaqMan qPCR assay was highly sensitive and specific and constitutes a useful molecular tool for detection and quantification of Pb in soil, plants and water. Thus, the assay is proposed as an effective and efficient tool for early diagnosis of Pb to support quarantine measures and disease risk assessment, limiting the impact and spread of the pathogen.

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