

Detection of Quarantine Pathogen *Pectobacterium caratovorum* in Potato Seed Lots Imported to Sri Lanka

Rajapaksha R.W.P.M. *, Vivehananthan K. *, Warshamana I.K. **

* Department of Biotechnology, Faculty of Agriculture & Plantation Management, Wayamba University of Sri Lanka

**National Plant Quarantine Service, Canada Friendship Road, Katunayake, Sri Lanka

Abstract- The majority of countries today follow stringent quarantine regulations to prevent the entry of pests into their countries especially with the seed stocks. *Pectobacterium caratovorum* is one of the significant bacterial pathogens of various horticultural crops around the world and a quarantine pathogen of seed potato in Sri Lanka. This research focused on detection of quarantine pathogens associated with imported seed potato consignments. Seed potato samples were collected from the intercepted consignments at the entry ports. Microbial DNA extraction protocol was optimized. Extracted DNA was sequenced using Ion Torrent Next Generation Sequencing technology by amplifying 400 bp V1-V2 region of 16S rRNA gene for the detection of bacterial pathogens. The recovered sequences were aligned with 16s rDNA in NCBI, Greengenes and SILVA databases for the detection of bacteria associated with the samples. Further, sub species level of the quarantine pathogen was identified by PCR amplification. The optimized DNA extraction protocol enabled faster isolation of microbial DNA from seed potato tubers and DNA was sufficient for PCR amplification and Next Generation Sequencing (NGS) analysis. Out of twenty four samples analyzed by NGS, the quarantine pathogen *Pectobacterium caratovorum* was detected only in one sample. In addition, subspecies level was confirmed as *Pectobacterium caratovorum* subsp. *caratovorum*. As this pathogen is the causal organism of soft rot and black leg diseases of potato, detection of the pathogen in asymptomatic potato tubers indicate the risk of latent infections associated with the imported seed lots.

Index Terms-Next Generation Sequencing, Quarantine, *Pectobacterium caratovorum*, Seed potato

I. INTRODUCTION

Diseases can drastically reduce the yield and quality of potato tubers therefore, relative freedom from pathogenic microbes is of major importance in potato production. However, potato plant and its tubers are vulnerable to many diseases and multiple pathogenic microbial species present within infested tubers [12].

Detection of microbial pathogens is one of the most critical quarantine operations as plant pathogens belong to very diverse groups. Certain pathogens cause visible symptoms distinct enough for quick disease identification. However, in many situations, visual appearance is not clear enough for exact diagnosis of the problem [5]. As a result, identification of the actual pathogen becomes a challenging task and a highly time consuming process. Therefore, the tools used for diagnosis purposes must be sensitive enough for accurate detection of the presence of pathogen nucleic acids even extremely in low amounts. In addition, it should provide sufficient specific information to identify the genetic variants of whatever pathogens available [1].

Generally, hybridization and amplification based molecular markers are powerful, reliable and offer advantages over morphological and biochemical alternatives of microbial detection [3]. PCR assay is superior to other techniques in its simplicity, rapidity and sensitivity in detection of a target DNA fragment [12]. However, it has been identified that the latent infections of many quarantine plant diseases had escaped from detection by routine diagnostic assays such as PCR or nucleic acid hybridization which are only suited for the identification of specific and well characterized pathogens [1].

Soft Rot Enterobacteriaceae (SRE) are recognized among the top 10 most important bacterial pathogens in agriculture. Of all *Pectobacterium* species infecting potato *Pectobacterium caratovorum* subsp. *caratovorum* has the widest host range worldwide [2]. *Pectobacterium caratovorum* subsp. *caratovorum* is polyphagous and therefore identification of these subspecies is important for potato growers in making adequate crop rotation choices to avoid field contamination problem. It is a serious pathogen of field plants causing soft rot and black leg diseases. In addition, *Pectobacterium caratovorum* subsp. *caratovorum* was identified as the responsible agent of stem rot and vascular wilt symptoms of hydroponically grown plants [6]. Tubers with latent infections will not develop the disease under suitable environmental conditions but anaerobic conditions in the field promote soft rot and black leg diseases in the crop [4]. Therefore, it is importantly essential for the farmers to use the pathogen free seed stocks for cultivation to prevent disease invasions. As *Pectobacterium caratovorum* subsp. *caratovorum* exists within the tuberosity lenticels or vascular system of potato tubers, the disease control methods under field conditions are unsuccessful [2]. Therefore, laboratory tests using reliable and sensitive molecular techniques for assessing seed tuber contamination incidence is important [9].

Next generation sequencing technology can potentially provide an ideal platform for identifying almost all known and unknown microbes present in any particular host [1]. The present study focused on application of 16s rDNA based approaches for microbial pest detection of seed potato imported to Sri Lanka using Ion Torrent Next Generation Sequencing (NGS). These studies lead the benefit of NGS for a large scale study of microbial populations by exploring the whole nucleotide sequence content of a sample.

II. MATERIALS & METHODS

A. Plant Material Collection

The seed potato tubers randomly sampled from the imported consignments at the entry ports of Sri Lanka were collected from the pathology division of National Plant Quarantine Service- Katunayake, Sri Lanka. The seed potato tubers within the consignments were not found to be infected with any quarantine important disease by visual observation. The collected seed tubers were separately stored in a cold room under 4 °C at the National Plant Quarantine Service.

B. Microbial DNA Extraction from the Imported Potatoes

The seed potato tubers were crushed and the extract was directly used for PCR amplification for pathogen detection. In parallel, the seed tuber parts especially from the stolon end and the eyes of potato tubers were ground manually and added into 15 ml of LB media. It was shaken at 120 rpm for 12 hrs. and centrifuged at 12400 g for 10 min to obtain the microbial pellet. The pellet was washed twice with a wash buffer (50 mM TrisHCl & 5 mM EDTA of pH 8.0) under 12400 g for 10 min and lysed with a lysis buffer (100 mM Tris HCl & 100 mM EDTA of pH 8.0, 1.5 M NaCl) with vortex for 5 min. The homogenized mixture was centrifuged at 12400 g for 15 min. The resulting supernatants were then centrifuged under 12400 g for 15 min with 75 µl of NaOAC and 1:1 ice-cold isopropanol added along the wall of the tube. The pellet was washed with 70% ethanol and re-centrifuged at 12400 g for 10 min. Ethanol was completely removed by air drying. The DNA was re-suspended in de-ionized water.

C. Ion Torrent Next Generation Sequencing and Analysis

The Ion PGM HiQ Sequencing Kit which includes reagents and materials for sequencing upto 400bp inserts was used together with Ion PGM HiQ template preparation kit compatible with Ion PGM HiQ Sequencing Kit. The Ion 318 Chip v2 was used on the Ion PGM System. DNA sequencing was performed on an Ion Torrent sequencing machine at Credence Genomics Pvt. Ltd. Colombo, Sri Lanka. Twenty four DNA samples were sequenced for bacterial identification. Hyper variable region of bacterial 16S rRNA gene was amplified using oligonucleotides, prepared into single-ended libraries of 400 bp size followed by Next Generation Sequencing on Ion Torrent PGM. Sequences of twenty four meta-genomic samples were recovered from ion torrent personal genome machine and aligned with the 16s rDNA of representative fragments. The DNA sequences were mapped to the NCBI-nt (26:10:2015), WGS (06:10:2014), Greengenes, Silva databases using QIIME version 1.9.0 and Credence Infectious Panel Pipeline 1.1.0.

D. PCR Amplification for Detection of Pathogen at the Subspecies Level

Based on the results obtained by NGS analysis, genomic DNA extracted from seed potato tubers were further screened with the species specific PCR to identify the quarantine pathogens upto sub species levels. *Pectobacterium caratovorum* subsp. *caratovorum* specific 312bp DNA fragment was amplified for the confirmation of the quarantine pathogen upto sub species level. PCR amplification was carried out using 1 µl of DNA template in a 20 µl reaction containing 1 µl of 10 µM primers (Ec001 F: 5'-CGGTTACGATCAGCGTCTCG-3' and Ec001R 5'- GATGTGCCGATGCCGATAC-3') and GoTaq Hot Start Master Mix (Promega) following the manufacturer's protocol. The following cycling conditions were used: initial denaturation of 95 °C for 5 min, 30 cycles of 94 °C for 30 sec, 62°C for 30 sec, 72 °C for 30 sec. An additional final extension for 10 min at 72 °C was then performed.

III. RESULTS

A. Use of Crushed Potato Extract and DNA from Potato Tubers for Analysis

The application of PCR in seed tuber analysis is restrained by the time consuming extraction of microbial DNA from potato tubers. However, it has been identified that direct use of crushed potato extract is ineffective in PCR amplification. The DNA isolation method optimized in this study yielded microbial DNA from the seed potato tubers in satisfactory amounts (**Figure 1**). The quality and quantity of DNA obtained by using this simple DNA extraction protocol was indicated by successful PCR amplification and NGS. The DNA obtained by using the optimized extraction protocol could be free of co-extracted PCR inhibitors which resulted the successful 16s rRNA amplification (**Figure2**).

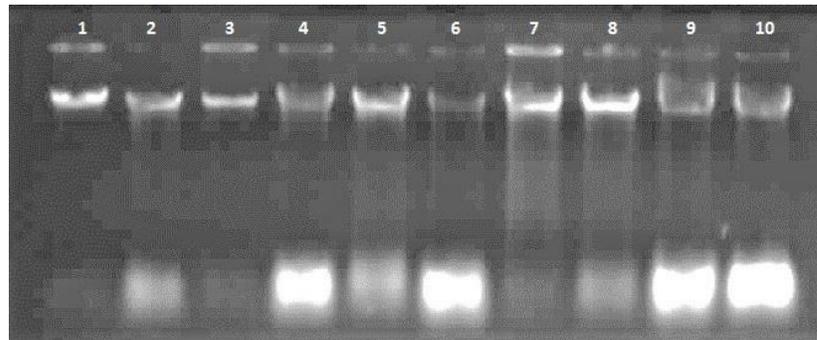


Figure 1: DNA extracted from seed potato samples. Lane 1-10: DNA samples

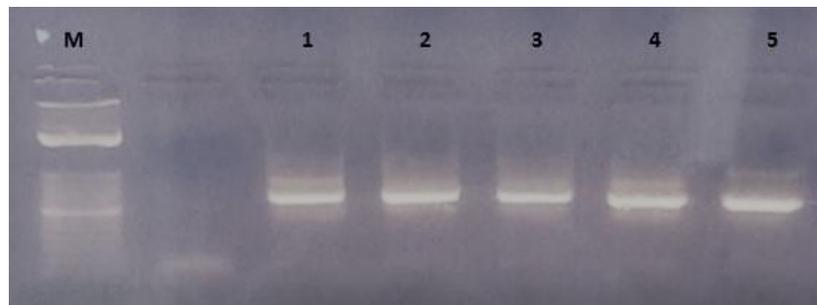


Figure 2: Amplification of V1-V2 region of 16s rDNA from Microbial DNA extracted from seed potato M: 100bp Marker; 1,2,3,4: DNA samples; P: Positive control

B. NGS Analysis of Bacterial Populations

Ion Torrent Next Generation Sequencing analysis generated number of microbial endophytes colonized within potato tissues. Average 16-80 bacterial species per sample were detected by NGS which revealed a greater prevalence and diversity of microbes in the analysed seed potato. The sequence analysis resulted the presence of quarantine pathogens *Pectobacterium caratovorum* only in one analysed sample. Together with the quarantine pathogen, this sample contained high proportions of microbes from the species *Staphylococcus xylosus* and *Staphylococcus succinus*. The other detected species *Raoulterra planticola*, *Leuconostoc mesenteroides*, *Lactococcus lactis*, *Citrobacter freundii* were also identified as abundant genera present in the seed potato sample. Furthermore, the quarantine pathogen *Pectobacterium caratovorum* was detected under 0.2% low abundance level. However, the detection of low abundance microbes enabled identification of asymptomatic latent infections within the sample (**Table 1**). Maximum likelihood phylogenetic trees were inferred for the 16S rDNA fragments (Data not shown).

Five common bacterial phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Spirochetales* were detected in all analysed 24 samples. Among them, the abundance of bacteria belonging to the phyla *Proteobacteria* was exceeding 50% which included *alpha*, *beta*, *gamma* and *delta* *proteobacteria* species in high abundance.

C. PCR analysis of the DNA with the primers specific for *Pectobacterium caratovorum* subsp. *caratovorum*

Pectobacterium caratovorum identified by NGS was confirmed for the detection of subspecies level as *Pectobacterium caratovorum* subsp. *caratovorum* using the specific primers. The amplification was reproducible and revealed one major product of 312 bp in length. No amplification of target sequence was observed in negative samples (**Figure 3**).

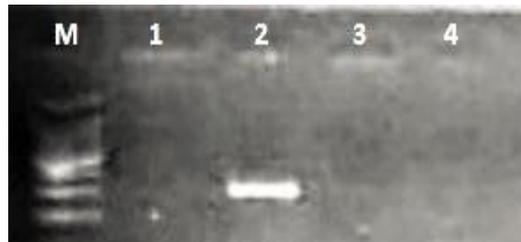


Figure 3: Amplification of seed potato DNA with *Pectobacterium carotovorum* subsp. *carotovorum* specific primers M: 100bp Marker; Lane 1-4: Seed potato samples

Table 1 : Identified bacteria in one sample of imported seed potato by Next Generation Sequencing Analysis.

| Species | Relative Abundance |
|---------------------------|--------------------|
| Staphylococcus xylosus | 79.61 % |
| Staphylococcus succinus | 12.55 % |
| Raoultella planticola | 2.16 % |
| Leuconostoc mesenteroides | 1.57 % |
| Lactococcus lactis | 1.18 % |
| Citrobacter freundii | 0.59 % |
| Brenneria rubrifaciens | 0.2 % |
| Enterobacter kobei | 0.2 % |
| Enterobacter sp. 638 | 0.2 % |

| Species | Relative Abundance |
|-----------------------------|--------------------|
| Enterococcus avium | 0.2 % |
| Ewingella americana | 0.2 % |
| Hafnia psychrotolerans | 0.2 % |
| Pantoea anthophila | 0.2 % |
| Pectobacterium carotovorum | 0.2 % |
| Staphylococcus gallinarum | 0.2 % |
| Staphylococcus haemolyticus | 0.2 % |
| Yersinia kristensenii | 0.2 % |
| Yersinia mollaretii | 0.2 % |

DISCUSSION

This study revealed significant associations of microbes in the imported seed potato consignments to Sri Lanka. The attempts initially taken to use the crushed potato extract for direct PCR amplification was ineffective. The decreased sensitivity could be attributed to PCR interfering compounds present within the potato extract and the low abundance of bacteria in the sample. In latent infection, the bacteria may be found in all parts of tissues however, their density is usually low. When the pathogen populations are low, they need to be enriched above detection level. Most methods used for identification of soft rot and back leg causing bacteria require isolation of viable cells from samples and purification of bacteria prior to analysis [2]. In the present study, initial incubation of crushed potato with liquid LB acts as a common enrichment medium for bacteria available within the potato tissues to increase bacterial cell number. This enrichment medium creates conditions that stimulate growth and multiplication of bacteria possibly facilitating the detection of all available pathogens within the sample including the endophytes. It is similarly important as microbial endophytes that colonize potato tissue are widespread and some of the non-pathogenic endophytes could also turn into phytopathogens that are able to induce infection symptoms [11]. Therefore, developed protocol allows multiplication of available pathogens which possibly enables isolation of most of the bacteria in the samples.

In potato tubers, microbes are more commonly present in the stolon end and frequently found in the lenticels and suberized wounds [2]. Therefore, tissues especially from the stolon end was taken for analysis. In many protocols an antioxidant such as DIECA (diethyldithiocarbamic acid) is used in very low concentrations (0.05%) during enrichment to protect bacteria cells from oxidative stress due to the release of the plant compounds [2]. However, any chemical was not applied in this extraction protocol to remove the

phenolic contaminants. The DNA extraction protocol developed in this study was found to be very much effective to obtain quality DNA in sufficient amounts. The extracted DNA was sensitive enough for both PCR and NGS analysis.

Molecular detection studies for bacteria are commonly performed by analyzing the prokaryotic 16S ribosomal RNA gene (16S rRNA) which is approximately 1,500 bp long and it contains nine variable regions interspersed between conserved regions. The variable regions of 16S rRNA are frequently used in phylogenetic classifications such as genus or species levels in diverse microbial populations [10]. Ion Torrent next generation sequencing of 400bp fragment of V1-V2 region of bacterial 16S rDNA was quite reproducible. As expected, greater numbers of species-level classifications were obtained where average 20 - 80 species per sample were detected. In addition, NGS technology based on 16S rRNA analysis showed higher sensitivity to detect low prevalence bacteria within the samples. The relative abundance level of the identified quarantine pathogen *Pectobacterium caratovorum* was 0.2%. However, Ion Torrent NGS analysis failed to detect the pathogen upto subspecies levels. NGS sequencing experiments produce millions to billions of short sequence reads at a high speed [7] but unable to amplify more than two or three fragments of the nine variable regions of 16s rRNA gene which may affect the detection of sub species levels.

NGS has currently gained increasing attention in the field of plant pathology. However, analysis of microbes using this technology remains computationally challenging and both time and resource intensive, making the approach prohibitive in the routine plant pathogen diagnostic assays [8]. Ion Semiconductor next-generation sequencing (Ion Torrent) offers rapid chemistries that make it amenable for adaptation as a plant pathogen diagnostic tool, so it was selected as the sequencing platform in this study. In addition, the 16S rDNA sequence mapped with four different databases; NCBI-nt (26:10:2015), WGS (06:10:2014), Greengenes and Silva with 98-100% similarity verifying the reliability of data analysis and providing the level of accuracy for quarantine diagnosis.

CONCLUSIONS

The risk of latent infections of quarantine pathogen *Pectobacterium caratovorum* subsp. *caratovorum* was identified in imported seed potato consignments. The developed simple DNA extraction method and amplification based detection by using NGS can be successfully employed in analyzing latent infections of seed potato tubers in plant quarantine diagnosis activities.

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AUTHORS

First Author – R.W.P.M. Rajapaksha, MPhil. student, Dept. of Biotechnology, Faculty of Agriculture & Plantation Management, Wayamba University of Sri Lanka piumi.rajapakse25@gmail.com.

Second Author – K. Vivehananthan, , Head/ Dept. of Biotechnology, Faculty of Agriculture & Plantation Management, Wayamba University of Sri Lanka kalaivanivive@gmail.com.

Third Author – I.K. Warshamana, Assistant Director of Agriculture, National Plant Quarantine Service, Katunayake- Sri Lanka

Correspondence Author – R.W.P.M. Rajapaksha piumi.rajapakse25@gmail.com +94 0717776805

