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Development of Polyclonal Rabbit Serum-Based ELISA for Detection of *Pectobacterium carotovorum* subsp. *carotovorum* and its Specificity against other Causing Soft Rot Bacteria

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ABSTRACT

The soft rot bacteria *Pectobacterium* and *Dickeya* species cause economically important diseases on potato crops. Specific and efficient detection methods are essential to investigate the ecology and pathogenesis of the soft rot bacteria as well as in seed certification programmes. Polyclonal antibodies against potato soft rot bacterium; *Pectobacterium carotovorum* subsp. *carotovorum* were generated in female New Zealand white rabbits. Determination of the optimum period to collect the antiserum (As) using indirect Enzyme-Linked Immunosorbent Assay (ELISA) showed that, the first collecting date 7 days after the last injection was the best followed by the second collecting date 14 days in both killed (As1) and live (As2) bacterial antisera. The best positive values of antiserum titer were obtained with As1 (7 days) up to dilution of $1:1.28 \times 10^4$ then As1 (14 days) $1:6.4 \times 10^3$ and As2 (7 or 14 days) $1:8 \times 10^2$. Efficiency of the antiserum to comparing among six isolates causing soft rot, *Pectobacterium carotovorum* subsp. *carotovorum* (PCCS63) which used for antisera production, *P. carotovorum* subsp. *carotovorum* (IPO1949), *P. carotovorum* subsp. *atrosepticum* (1007), *P. carotovorum* subsp. *wasabiae* (ipp041), *Dickeya dianthicola* (IPO2114) and *Dickeya chrysanthemi* (DSM4610) revealed that, our polyclonal antiserum had a high sensitivity to react with *P. carotovorum* subsp. *carotovorum* isolates PCCS63 and IPO1949 than with *D. chrysanthemi* DSM4610 isolate and the least coupling was found with *P. carotovorum* subsp. *wasabiae* ipp041 and *Dickeya dianthicola* IPO2114 isolates. The produced antiserum was more sensitive to detect *P. carotovorum* subsp. *carotovorum* isolates than the other soft rot bacteria.

Key words: *Pectobacterium carotovorum*, soft rot, polyclonal antibodies, antiserum, ELISA

INTRODUCTION

Contamination of seed tubers with soft rot bacteria is one of the biggest problems in seed potato production. These bacteria cause blackleg, rotting of potato stems in the field and soft rot of tubers during storage. Seed lots are discarded frequently due to blackleg in the field or soft rot in storage. Latent infections in seed tubers often result in blackleg appearing unpredictably in the field. Furthermore, these bacteria also cause severe storage losses for the potato processing industry, especially in large warehouses without refrigeration facilities. In temperate climates

Pectobacterium carotovorum subsp. *carotovorum* (Pcc) (formerly, *Erwinia carotovora* subsp. *carotovora*; Gardan *et al.*, 2003), has been regarded as the causal agent of soft rot (Perombelon, 2002; Toth *et al.*, 2003) while *Pectobacterium atrosepticum* (formerly, *Erwinia carotovora* subsp. *atroseptica*; Gardan *et al.*, 2003) has been typically considered to cause both blackleg in the field as well as soft rot during storage.

Dickeya spp. (formerly, *E. chrysanthemi* and *P. chrysanthemi*, Samson *et al.*, 2005) have long been recognized as pathogens of ornamental plants particularly in tropical and subtropical climates. In warm climates, *Dickeya* has also been reported to cause both blackleg and soft rot of potato (Perombelon, 2002). *Dickeya* strains have been isolated repeatedly from potatoes during recent decades and the disease has rapidly spread in Western Europe during recent years and Africa (Palacio-Bielsa *et al.*, 2006; Behiry, 2013). Variability within the former species *Erwinia chrysanthemi* is large and the strains were divided into several biovars and pathovars (Boccarda *et al.*, 1991; Nassar *et al.*, 1996; Avrova *et al.*, 2002). The variability was studied with different methods, such as PCR amplification and sequencing or RFLP of 16S or 16S-23S rDNA intergenic spacer (Toth *et al.*, 2001; Fessehaie *et al.*, 2002) and ELISAs (Alarcon *et al.*, 1995; Duarte *et al.*, 2004).

Enzyme Linked Immuno Assay (ELISAs) is one of the most sensitive and frequently used methods for estimation of pathogens (i.e., bacteria) and Lipopolysaccharide (LPS) molecules. Thus it may be possible for ELISAs to detect low levels of soft rot *Pectobacteria*. However, sensitivity was limited to 10^7 CFU mL⁻¹, in ELISA compared to a sensitivity of 10^3 CFU mL⁻¹ for a PCR test using published primers directed to the pectate lyase gene (Nassar *et al.*, 1996). An enrichment ELISA procedure has been used in the Netherlands for routine detection of *Dickeya* spp. include cost efficiency and a correlation of 95% between this method and PCR testing (Van Der Wolf *et al.*, 2006). Whole bacterial cells (i.e., Pcc) and Lipopolysaccharide (LPS), the major surface component of gram-negative bacteria specifically implicated in plant pathogenesis, are major antigens capable of initiating an immune response in various animal hosts. (Bassoriello, 2010) developed polyclonal ELISAs antibodies against *P. carotovorum* subsp. *carotovorum* Lipopolysaccharide (LPS) and Heat-killed (HK) or live *P. carotovorum* subsp. *carotovorum* (Pcc) limited the detection and quantification of the pathogen. The objectives of this work aimed to develop and characterize polyclonal rabbit serum raised against heat-killed *P. carotovorum* subsp. *carotovorum* and *P. carotovorum*. subsp. *carotovorum* LPS to determine which elicits a more specific response to *P. carotovorum* subsp. *carotovorum*; determine whether cross-reactivity exists among antibodies specific to *P. carotovorum* subsp. *carotovorum* and *Pectobacterium* spp. or other related genera that may be cause soft rot or blackleg of potato and develop a polyclonal rabbit serum-based ELISA for detection of *P. carotovorum* subsp. *carotovorum* and other soft rot pathogens.

MATERIALS AND METHODS

Antiserum production: Suspensions (ca. 10^8 CFU mL⁻¹ in phosphate buffer saline, PBS) of heat killed, live cells from soft rot bacterial isolate *P. carotovorum* subsp. *carotovorum* PCCS63 previously identified by biochemical and molecular tests (Behiry, 2013) and lipopolysaccharides of *P. carotovorum* (Pcc LPS) and *P. atrosepticum* (Pca LPS) (provided by Dr. Mammdoh Ismail, Fac. of Agric., El-Menia, Egypt) were used to produced antisera in four female New Zealand white rabbits by five injections course, (0.5, 1.0, 1.5, 2.0 and 2.5 mL, respectively) the first one subcutaneous and the others in muscle thigh with 7 days interval (Al-Ani *et al.*, 2004).

Bleeding from the marginal ear vein was carried out after 7 and 14 days from the last injection and left for clotting 2-3 h at room temperature then stored over night at 4°C. Antisera were clarified by centrifugation at 5000 rpm for 10 min and stored in the presence of 0.05% sodium azide at -20°C till used (Kayali *et al.*, 2004). Normal serum was obtained from rabbit before immunization to use as a control in subsequent tests.

Indirect ELISA

Determination of the optimum antiserum collecting period: Indirect ELISA was carried out as described by Younes (1995). Bacterial suspension was prepared in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.2) at a concentration of ca. 10^8 CFU mL⁻¹. Wells were coated by adding 100 µL of the bacterial suspension to the bottom of the well (2 wells per each antiserum dilution) and incubated over night at 4°C. The plates were rinsed three times with washing solution PBS -Tween 20 for 3 min each.

Eight dilutions of double fold up to 1:12800 for normal serum (as control) and the two antisera obtained after 7 and 14 days of the last injection in serum buffer (PBS-Tween 20 containing 2% soluble polyvinylpyrrolidone, 0.2% BSA) were used. One hundred microliter aliquots from the diluted normal serum and antisera were added to each well, after which the plates were incubated at 37°C for 2 h and then washed as before.

Goat anti-rabbit gamma globulin conjugated to alkaline phosphatase (Sigma No 2937) was diluted 1:20000 in serum buffer and 100 µL were added to each well and incubated at 37°C for one hour and then washed as before.

One hundred microliter of the enzyme substrate, 0.5 mg mL⁻¹ para-nitrophenyl phosphate in 10% diethanolamine buffer, pH 9.8 were added to each well and incubated at room temperature (25°C) for about 30 min. The enzyme activity was stopped by adding 50 µL of 3M NaOH. ELISA values measured by ELISA reader and expressed as absorbency at 405 nm and absorbency values at least double that of the healthy control (normal serum) were considered positive.

Antiserum titer: The titer of *P. carotovorum* subsp. *carotovorum* PCCS63 antiserum was determined by using indirect ELISA. Bacterial suspension was prepared in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) at a concentration of ca. 10^8 CFU mL⁻¹. Wells were coated by adding 100 µL of the bacterial suspension to the well (2 wells per each antiserum dilution). Serial dilutions of double fold up to $1:1.28 \times 10^4$ of antiserum in serum buffer were used. The absorbance values were measured at 405 nm.

Determination of antiserum efficiency: Indirect ELISA previously described was used to demonstrate the sensitivity of polyclonal antisera to differentiate among all soft rot bacterial isolates; two isolates of *P. carotovorum* subsp. *carotovorum* and four isolates from *Enterobacteriaceae* related genera as follows; *P. carotovorum* subsp. *carotovorum* PCCS63 (used for production antisera), *P. carotovorum* subsp. *carotovorum* (IPO1949), *P. carotovorum* subsp. *atrosepticum* (1007), *P. carotovorum* subsp. *wasabiae* (ipp041), *D. dianthicola* (IPO 2114) and *D. chrysanthemi* (DSM4610) through their reaction with the produced antiserum against PCCS63 isolate (kindly provided by Prof Dr. Van der Wolf, Plant Research International, Wageningen, Netherlands).

RESULTS

Antisera production confirmation: Results of antiserum production indicated in Table 1 showed that, killed or live bacterial cells of isolate PCCS63 were only positive and interact with homologous antiserum which appeared in ELISA readings. On the other hand, antisera of Pcc LPS and Pca LPS failed to show any relation or positive reaction with PCCS63 cells in ELISA test as compared with normal serum (control).

Optimum antiserum collecting period: Results of indirect ELISA to determine the optimum period to collect the antiserum showed that the first collecting date 7 days after the lasted injection was the best followed by the second collecting date 14 days in both killed and live bacterial antisera Table 2.

Determination of antiserum titer: The best positive ELISA values were obtained with As1 (7 days) up to dilution of 1:1.28×10⁴ then As1 (14 days) 1:6.4×10³ and As2 (7 or 14 days) 1:8×10² Table 2.

Efficiency of the antiserum: Indirect ELISA was used for comparing among six isolates causing soft rot and from *Pectobacterium* related genera: *P. carotovorum* subsp. *carotovorum* PCCS63 (used for production antisera), *P. carotovorum* subsp. *carotovorum* IPO1949, *P. carotovorum* subsp. *atrosepticum* 1007, *P. carotovorum* subsp. *wasabiae* ipp041, *D. dianthicola* IPO2114 and *D. chrysanthemi* DSM4610. Data presented in Table 3 revealed that our polyclonal antiserum could differentiate among the soft rot bacterial isolates and exposed that a relation among these isolates, in the same way, our polyclonal antiserum had a high sensitivity to react with PCCS63 and IPO1949 isolates, less with DSM4610 isolate and finely the least coupling with ipp041 and IPO2114 isolates.

Table 1: Indirect ELISA absorbance values (405 nm) of *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) cells and lipopolysaccharides of *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *atrosepticum* (Pca) in its antiserum

Antiserum collection (7 days)				
Normal serum	Pcc LPS	Pca LPS	Pcc live	Pcc killed
0.102	0.105	0.056	2.017	1.926

ELISA absorbance values at 405 nm are average of two replicates each, absorbency values of at least double that of the healthy control were considered positive

Table 2: Indirect ELISA absorbance values (405 nm) of *Pectobacterium carotovorum* subsp. *carotovorum* PCCS63 in various dilutions of its antiserum with different collection periods

Serum dilution	Normal serum	Antiserum			
		7 days		14 days	
		As 1	As 2	As 1	As 2
1:10 ²	0.101	0.3075	0.1945	0.2750	0.1910
1:2×10 ²	0.087	0.2565	0.1705	0.2580	0.1915
1:4×10 ²	0.084	0.2525	0.1660	0.2490	0.1805
1:8×10 ²	0.075	0.2135	0.1565	0.2360	0.1685
1:1.6×10 ³	0.082	0.2075	0.1440	0.2150	0.1415
1:3.2×10 ³	0.074	0.1975	0.1260	0.1880	0.1225
1:6.4×10 ³	0.068	0.1640	0.1230	0.1390	0.1180
1:1.28×10 ⁴	0.068	0.1400	0.1190	0.1350	0.1165

ELISA absorbance values at 405 nm are average of two replicates each, absorbency values of at least double that of the healthy control were considered positive, As 1: Antiserum produced from Pcc live cells, As 2: Antiserum produced from Pcc killed cells

Table 3: Indirect ELISA absorbance values (405 nm) of *Pectobacterium carotovorum* subsp. *carotovorum* and related *Enterobacteriaceae* in various dilutions of its antiserum

Serum dilution	Normal serum	Bacterial isolates (ca. 1×10^8 CFU mL ⁻¹)					
		Pcc 1	Pcc 2	Pca 3	Pcw 4	Ddin 5	Dch 6
1:10 ²	0.212	1.146	0.824	0.961	0.444	0.532	1.104
1:2×10 ²	0.198	0.999	0.795	0.812	0.297	0.380	1.039
1:4×10 ²	0.160	0.917	0.704	0.728	0.229	0.314	1.019
1:8×10 ²	0.123	0.908	0.702	0.651	0.176	0.217	0.995
1:1.6×10 ³	0.121	0.920	0.627	0.626	0.142	0.180	1.051
1:3.2×10 ³	0.112	0.749	0.536	0.593	0.119	0.138	0.812
1:6.4×10 ³	0.109	0.703	0.444	0.408	0.109	0.132	0.482
1:1.28×10 ⁴	0.109	0.574	0.311	0.307	0.105	0.124	0.281

ELISA absorbance values at 405 nm are average of two replicates each, absorbency values of at least double that of the healthy control were considered positive, Pcc 1: *P. carotovorum* subsp. *carotovorum* PCCS63, Pcc 2: *P. carotovorum* subsp. *carotovorum* IPO1949, Pca 3: *P. carotovorum* subsp. *atrosepticum* 1007, Pcw 4: *P. carotovorum* subsp. *wasabiae* ipp041 (HQ424871), Ddin 5: *Dickeya dianthicola* IPO 2114, Dch 6: *Dickeya chrysanthemi* DSM4610

DISCUSSION

Serological tests have been used to screen seed potatoes for latent populations of *Pectobacterium* spp. and *Dickeya* spp., but have generally been found to lack the required specificity and sensitivity. However, results of indirect ELISA to determine the optimum period to collect the antiserum showed that the first collecting date (7 days after the last injection) was the best followed by the second (14 days) so, it could be concluded that the efficiency of antiserum decreased as the collection period advanced. This is in contrast with Mohamed *et al.* (2008) and contract with those of Hamwih *et al.* (1999) and Kayali *et al.* (2004) thus, could be attributed to genera, species and types of the pathogenic bacteria involved in the tests.

ELISA positive antiserum titer values were obtained up to dilutions of 1:1.28×10⁴. These results were in agreement with those of Alarcon *et al.* (1995), Hamwih *et al.* (1999), Kayali *et al.* (2004) and Mohamed *et al.* (2008) who mentioned that, it is important to determine the antiserum titer to reduce its consumption.

According to our results about the efficiency of collected antiserum to detect and differentiate among all tested soft rot isolates, there was a weak reaction between the antiserum and the both strains *P. wasabiae* and *D. dianthicola*, this was in the same way of the findings of van Der Wolf *et al.* (1993), who found false positive results and limitations regarding the sensitivity of detection also remain a problem for serological detection of *Dickeya* spp. Such antibodies recognize only 68% of isolates (Samson *et al.*, 1990). While, a specify of a monoclonal antibody (6A6) to a fimbrial antigen detected all *D. dianthicola* isolates tested and some other *Dickeya* spp. than the polyclonal antibody in a Triple Antibody Sandwich (TAS) ELISA (Singh *et al.*, 2000) as well as, (Vernon-Shirley and Burns, 1992) produced three monoclonal antibodies, which reacted specifically with *P. carotovorum* and react successfully with 34 serogroup of *Pectobacterium atrosepticum* and in different results (De Boer *et al.*, 1979, 1987) *P. carotovorum* subsp. *carotovorum* isolates are serologically more diverse than *P. carotovorum* subsp. *atrosepticum* isolates in contrary with Jones *et al.* (1993) who couldn't differentiate between them using polyclonal antibodies. Serological techniques, including the use of monoclonal or polyclonal antibodies can be applied in the case of massive contaminations, but latent infections with low concentration of the pathogen (below 10⁵CFU mL⁻¹) require a more sensitive and reliable method to detect the bacterium in a field sample.

In general, the serological assays do not require expensive laboratory equipment, therefore they can be used everywhere, where simple, cheap and relatively sensitive and specific detection on a large scale is desirable, especially under field conditions.

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