Monoclonal Antibodies to the Lipopolysaccharide of *Erwinia carotovora* subsp. *atroseptica* Serogroup I

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ABSTRACT

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Monoclonal antibodies were produced to an *Erwinia carotovora* subsp. *atroseptica* serogroup I strain using outer membrane and soluble antigen extracts as immunogens. Monoclonal antibodies from three different hybridomas reacted with purified lipopolysaccharide. Furthermore, immunoblot analysis of lipopolysaccharide separated on polyacrylamide gels indicated that all three monoclonals reacted with the O-side chain. In immunofluorescence tests, the monoclonals reacted with all 18 *E. c.* subsp. *atroseptica* serogroup I strains tested. They also reacted with strains in serogroup XXII, but not with strains in serogroups XVIII and XX. They did not react with any of the 36 *E. c.* subsp. *carotovora* or eight *E. chrysanthemi* strains that were tested. One of the monoclonals was used for the enzyme-conjugate in enzyme-linked immunosorbent assays (ELISA), but this monoclonal did not work for coating the plates. Reaction of bacteria with the monoclonal-enzyme conjugate, however, was considerably lower than reaction with a comparable polyclonal-enzyme conjugate. Nevertheless, the monoclonal could be used in ELISA to differentiate between blackleg-infected and healthy potato stems. Blackleg contamination of symptomless tubers could not be detected by ELISA using the monoclonal-enzyme conjugate.

Additional key words: Detection, diagnosis, serology.

Erwinia carotovora subsp. *atroseptica* (van Hall) Dye is the major causal agent of the blackleg disease of potato. *E. c.* subsp. *atroseptica* is often present on seed tubers, but actual occurrence of the disease in the field depends on environmental factors (14). Control of blackleg by inspection and certification of seed potato crops has been only partially successful because of erratic symptom expression and the presence of other inoculum sources (8,9). A postharvest test for *E. c.* subsp. *atroseptica* would provide an actual measurement of seed tuber contamination.

Serological testing of seed tubers seems feasible since the disease is usually caused by strains of a single serogroup, serogroup I (5). In British Columbia, for example, 96% of *E. c.* subsp. *atroseptica* strains were serogroup I (S. H. De Boer, *unpublished data*). An immunofluorescence procedure in which tests are made on tubers induced to decay can be used if the number of samples is relatively small (1,20). Vruggink (19) has also developed an enzyme-linked immunosorbent assay (ELISA) for determining the presence of contamination of symptomless potato tubers by *E. c.* subsp. *atroseptica*. Although results with these tests have been encouraging, they have not been reliable enough for use in certification.

We considered the possibility of using monoclonal antibodies, rather than a polyclonal antiserum, to improve postharvest serological detection of *E. c.* subsp. *atroseptica* on seed potato tubers. Monoclonal antibodies have the advantage over polyclonal antisera in serological tests in that they react with a single epitope rather than with many different antigens. Thus, it is possible to obtain monoclonals of much higher specificity for a particular bacterium than with polyclonal antiserum. If a unique antigen of a pathogen has been defined, monoclonals can be selected that react only with it and thus will be specific for that pathogen. Furthermore, different preparations of any one monoclonal all have identical specificities, whereas specificity of polyclonal antisera may vary between preparations.

Lipopolysaccharide (LPS) is a major antigenic component of Gram-negative bacteria and is the determinant utilized in the serogrouping of *E. carotovora* (4,5) as well as many other enterobacterial species. Thus, an assay procedure based on detection of LPS may be useful for diagnosis of potato blackleg and detection of the pathogen in symptomless seed potato tubers. In this paper, we report the production of monoclonal antibodies to the LPS of *E. c.* subsp. *atroseptica* serogroup I and present preliminary data on the use of the monoclonal antibodies for detecting the pathogen on potato.

MATERIALS AND METHODS

Preparation of antigens. *E. c.* subsp. *atroseptica* strain 31, serogroup I, was used as the source of antigen preparations. Outer cell membrane fractions were obtained from cells grown in nutrient broth plus 1% glucose by the LiCl extraction procedure of Yakrus and Schaad (21). Another antigenic fraction was obtained as an 80% ammonium sulfate precipitate from 48-hr-old culture filtrate of cells grown in a sodium polypectate enrichment broth (11). LPS was purified from cells grown in Casamino acid/peptone/glucose (CPG) (1 g of Casamino acids, 10 g of Bacto-peptone, and 5 g of D-glucose per liter) broth as described previously (4).

Antibody preparation. Polyclonal antiserum was produced in a rabbit against glutaraldehyde-fixed cells (5). Monoclonal-antibody-secreting hybridomas were produced by the standard procedures we used previously (6). RBF/Dn mice were immunized interperitoneally with the other cell membrane fraction 14 days and again 3 days before fusion. BALB/c mice were immunized interperitoneally with the extracellular antigenic fraction at 7, 3, 2, and 1 wk and again 3 days before fusion. Splenocytes from an immunized RBF/Dn and a BALB/c mouse were fused with FOX-NY and Sp2/0-Ag14 myeloma cells, respectively, using polyethylene glycol as fusing agent.

The FOX-NY hybridomas were selected in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal calf serum (FCS) and a thymocyte feeder layer plus adenine-aminopterin-thymidine selection agents (17). The Sp2/0-Ag14 hybridomas were selected in

DMEM with 15% FCS and 5% horse serum but without thymocytes and containing the hypoxanthine-aminopterin-thymidine selection agents (10). Positive clones were identified by ELISA tests as described below using the corresponding antigens. Selected hybridomas were cloned two or three times by limiting dilution plating. Ascitic fluid was obtained 7–10 days after injecting hybridomas into pristane-primed mice.

Immunoglobulins (Ig) were purified from ascitic fluid on a Sephacryl S300 column measuring 85 × 2.6 cm in 0.01 M phosphate-buffered saline, pH 7.2 (PBS). Fractions with ultraviolet (260 nm) absorbance were collected and antibody activity determined by ELISA or immunofluorescence. The IgG fraction of the polyclonal rabbit antiserum was purified in the same way.

Immunoglobulin isotypes were determined by ELISA using hybridoma culture fluid and a hybridoma subisotyping kit (Calbiochem, San Diego, CA 92112).

Electrophoresis and immunoblotting. Electrophoresis of LPS preparations was carried out on a discontinuous system using a 4% acrylamide stacking gel and a 14% running gel without incorporating sodium dodecyl sulfate into the gels (16). Gels were run in a minivertical slab cell (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ont., Model 360) for 3.5 hr at 10 mA and then silver stained or transferred to nitrocellulose. Silver staining was done by the method described for proteins (12) except that LPS in gels was oxidized with 0.7% periodic acid in 40% ethanol and 5% acetic acid for 5 min rather than with sodium dichromate/nitric acid (18). LPS was transferred to nitrocellulose from the gels by blotting in a Bio-Rad trans-blot cell for 3 hr at 60 V with prechilled buffer (2). Before probing with the antibody, blots were blocked for 0.5 hr with 5% FCS in PBS and then treated overnight with purified antibody diluted 1:100 in PBS plus 5% FCS. After extensive washing in PBS, the blots were treated with alkaline phosphatase antimouse or antirabbit Ig conjugate (Bio/Can Scientific Inc., Mississauga, Ontario) at 1:1,000 in the PBS/FCS buffer for 2 hr. Finally blots were developed with substrate that was prepared with 10 mg of naphthol AS MX phosphoric acid (Sigma Chemical Co., St. Louis, MO 63178) and 20 mg of Fast Red TR salt (Sigma) per 10 ml of 50 mM Tris-HCl, pH 8.0 (16).

ELISA. Antigen preparations were coated onto polystyrene 96-well microtiter plates in 0.1 M carbonate buffer, pH 9.6, and incubated for 2 hr at 37 C or overnight at 4 C. Hybridoma culture fluids were tested directly on the coated plates, but purified immunoglobulins were diluted in PBS plus 2% FCS. The antibody preparations were incubated for 2 hr at 37 C and washed out before the addition of alkaline phosphatase conjugated antimouse Ig, which was diluted and incubated in the same way. *p*-Nitrophenyl phosphate tablets (Sigma) were used as substrate. Substrate conversion was quantified by measuring absorbance at 405 nm with a Titertek Multiskan MC plate reader.

To compare monoclonal with polyclonal antibodies in ELISA tests, conjugates of antibody and alkaline phosphatase were prepared by the glutaraldehyde procedure using 1 mg of antibody per 2.5 mg of enzyme (3). ELISA plates were coated with monoclonal or polyclonal antibody at 10 μg/ml (predetermined to be the optimum concentration) in carbonate buffer as described above. Whole bacterial cells washed from nutrient agar slants and adjusted to $OD_{660} = 0.1$ in PBS were used as antigen and incubated in coated plates for 2 hr at 37 C. After excess antigen was washed from plates, nonspecific protein binding sites were blocked by incubation with 5% FCS in PBS for 0.5 hr. Subsequently, monoclonal or polyclonal antibody-enzyme conjugate at various dilutions in PBS/FCS buffer was incubated in the plates for 1 hr at 37 C. Finally substrate was added and its development quantified as described above.

To test plant material, ELISA plates were coated with purified polyclonal antibody diluted to 10 μg/ml in carbonate buffer. Sap expressed from diseased potato stems was diluted in PBS and incubated in the coated plates for 2 hr at 37 C. After incubation with sap samples and blocking with FCS, plates were incubated with a 1:200 dilution of monoclonal antibody-enzyme conjugate for 1 hr at 37 C and then developed with substrate as usual. For potato tuber samples, a household potato peeler was used to obtain peel samples from which sap was extracted with a mechanical press and used directly in the test. The potato-peel sap samples were tested in ELISA the same way as the stem samples. Monoclonal-enzyme conjugate was diluted 1:200; polyclonal-enzyme conjugate, 1:1,000.

Immunofluorescence. For immunofluorescence tests, washed cells or plant sap samples were fixed directly to microscope slides and then stained by the indirect procedure (6).

RESULTS

Monoclonal antibodies. Two stable hybridoma cell lines (4F6 and 5E7) producing monoclonal antibodies to *E. c.* subsp. *atroseptica* were selected from the fusion in which the LiCl extract was used as antigen for both immunization and the ELISA for screening hybridomas; one (14E7) was selected from the fusion using the soluble antigenic fraction from the culture supernatant. All three monoclonals were IgG_3.

Reaction of monoclonal antibodies with LPS. The LPS from *E. c.* subsp. *atroseptica* strain 31 separated on gels into two or more bands depending upon the particular preparation. When more than two bands were present, the bands formed a ladder-rung pattern characteristic of LPS preparations from various other Gram-negative bacteria in silver-stained gels (Fig. 1, lane 1). All bands reacted with the rabbit polyclonal antisera on the blots (Fig. 1, lane 2). All bands except the lowest reacted with the monoclonals (Fig. 1, lane 3).

LPS purified from several *E. c.* subsp. *atroseptica* and *E. carotovora* subsp. *carotovora* (Jones) Bergey et al strains in different serogroups showed some differences in electrophoretic banding patterns (Fig. 2A). When the same LPS preparations were transferred from gels to nitrocellulose and probed using monoclonal 4F6, only the LPS preparations derived from serogroup I strains were stained well (Fig. 2B, lanes 1 and 5). Very

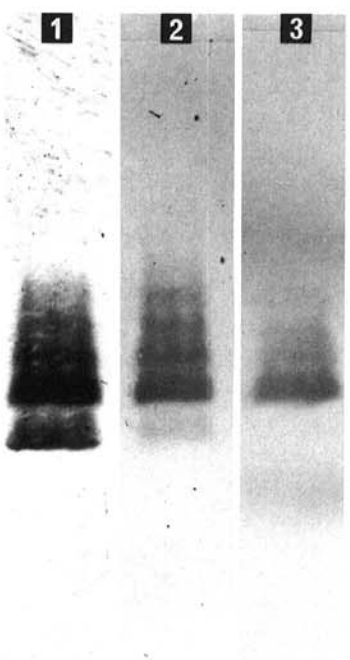

Fig. 1. Polyacrylamide gel electrophoresis profile of lipopolysaccharide from *Erwinia carotovora* subsp. *atroseptica* strain 31. Lane 1 is silver-stained gel. Lanes 2 and 3 are immunoblots of gels identical to lane 1 and probed with polyclonal antiserum and monoclonal antibody 4F6, respectively. Immunoblots probed with monoclonals 5E7 and 14E7 were identical to blot probed with 4F6.

weak staining occurred with LPS from a serogroup XXII strain (Fig. 2B, lane 8); good staining with this LPS was obtained when the amount applied to the gel was increased 10-fold (not shown). Increasing the other LPS concentrations on the gel 10-fold did not cause them to be stained.

Immunofluorescence. Specificity of all three monoclonals was found to be identical in immunofluorescence. They all reacted with the serogroup I and XXII strains, but with none of the other *E. c.* subsp. *atroseptica*, *E. c.* subsp. *carotovora*, or *E. chrysanthemi* Burkholder, McFadden, & Dimock strains (Table 1). Brightly fluorescing bacteria were observed in preparations from blackleg-diseased stems and tubers when monoclonal 4F6 was used for testing plant tissue samples by immunofluorescence.

ELISA with monoclonal antibodies. When monoclonal 4F6 was used for coating ELISA plates, it did not trap antigen applied subsequently to the plates (Fig. 3). However, the monoclonal could be used for the enzyme conjugate, although the intensity of final reaction was lower than when polyclonal-enzyme conjugate at an equal protein concentration was used (Fig. 3).

In ELISA, 4F6 discriminated between healthy and blackleg-infected stems. A_{405} values of diseased stems were about twice those of healthy stems (Fig. 4). Similar results were obtained with the polyclonal-enzyme conjugate; at comparable dilutions, however, A_{405} values were consistently higher than with monoclonal-enzyme conjugates, as was the case with pure cultures (Fig. 3). In ELISA tests with potato tubers, the A_{405} values with the monoclonal-enzyme conjugate did not show significant differences between tubers from healthy and infected plants, whereas the A_{405} values with polyclonal-enzyme conjugate were 10-fold higher with tubers from infected plants than with tubers from healthy plants (Table

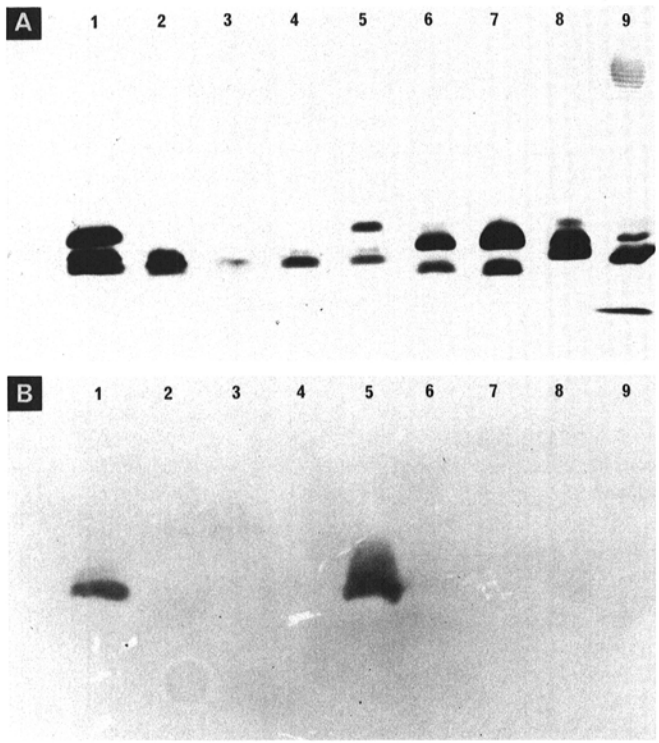

Fig. 2. Silver-stained polyacrylamide gel electrophoresis profiles **(A)** of eight *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *carotovora* lipopolysaccharide preparations (lanes 1–8) and *Escherichia coli* lipopolysaccharide (Sigma) (lane 9); and immunoblot **(B)** of identical gel probed with monoclonal 4F6. Lipopolysaccharide preparations used in **(A)** and **(B)** were from strain 31 serogroup I (lane 1), strain 6 serogroup XVIII (lane 2), strain 196 serogroup XX (lane 3), strain 198 serogroup XXII (lane 4), and strain 161 serogroup I (lane 5) of *E. c.* subsp. *atroseptica*; strain 21 serogroup II (lane 6) and strain 71 serogroup III (lane 7) of *E. c.* subsp. *carotovora*; and strain 565 serogroup XXII (lane 8) of *E. c.* subsp. *atroseptica*.

TABLE 1. Reaction in immunofluorescence test of monoclonal antibodies with strains of *Erwinia carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *carotovora*, and *E. chrysanthemi*

Strain	No. of strains tested	No. of strains positive		
		4F6	5E7	14E7
E. c. subsp. *atroseptica*				
Serogroup I	18	18	18	18
Serogroup XVIII	9	0	0	0
Serogroup XX	5	0	0	0
Serogroup XXII	5	5	5	5
E. c. subsp. *carotovora*	36	0	0	0
E. chrysanthemi	8	0	0	0

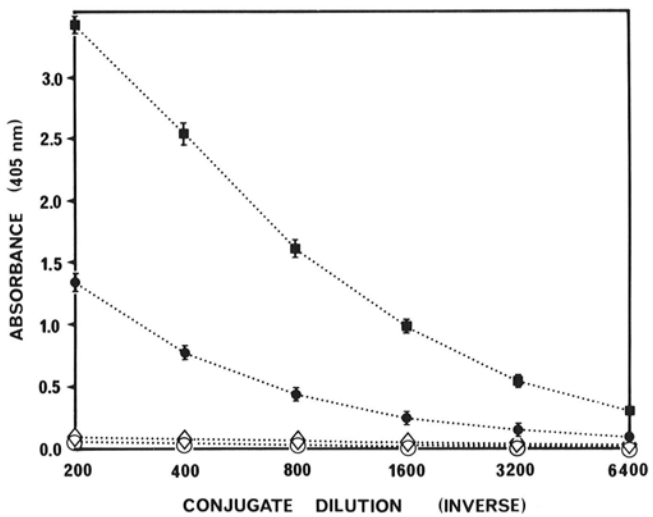

Fig. 3. Comparison of monoclonal 4F6 and polyclonal antisera in enzyme-linked immunosorbent assay using pure culture of homologous bacterium as antigen. Polystyrene plates were coated with polyclonal antisera and, after antigen loading, probed with monoclonal-enzyme (•) or polyclonal-enzyme (■) conjugates at six dilutions; or coated with monoclonal antisera and probed with monoclonal-enzyme (o) or polyclonal-enzyme (◇) conjugates. Data are shown as mean and standard error (vertical bars) of five determinations.

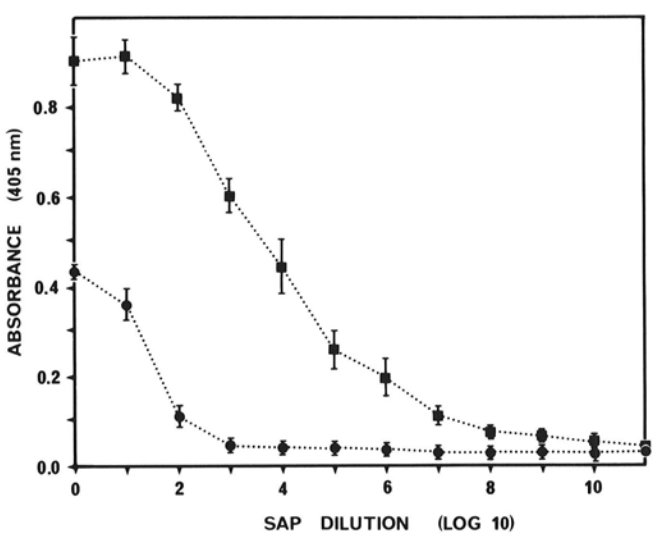

Fig. 4. Comparison of blackleg-infected (■) and healthy (•) potato stems in enzyme-linked immunosorbent assay using polyclonal antisera for coating plates and monoclonal 4F6-enzyme conjugate as probe. Data are shown as mean and standard error (vertical bars) of eight determinations.

2). However, A_{405} readings greater than 1.0 were obtained with the monoclonal-enzyme conjugate when randomly collected commercial tubers were tested.

DISCUSSION

Crude extracts were used as immunogens in this study because only low-antibody titers were obtained in mice immunized with purified LPS. However, monoclonal antibodies selected from both extracts reacted with LPS, which is a major antigenic component of the outer membrane of Gram-negative bacteria and was present in both of our antigen preparations.

Since *Erwinia* LPS is similar to that of other enteric bacteria (4,15), it is likely that *E. c.* subsp. *atroseptica* LPS will electrophorese in the same characteristic fashion as LPS from other bacterial species (16). Thus, the fastest migrating band on the silver-stained acrylamide gels (Figs. 1 and 2) probably represents the core region, and the remaining bands the O-side chain containing LPS units (7). Variation in the number of bands was probably the result of cleavage artifacts caused by the extraction process (13). The monoclonals reacted with the O-side chain containing units of the LPS but not with the core region, whereas the polyclonal antisera reacted with both the O-side chain and the core regions (Fig. 1).

The O-side chains are the principal antigenic determinants of enterobacterial LPS, and it is therefore likely that serogroup specificity is a function of this side-chain structure. Monoclonals against the LPS side chain would be expected to be highly serogroup specific, as was indeed the case.

In double-diffusion tests with polyclonal serogroup I antiserum, *E. c.* subsp. *atroseptica* strains of heterologous serogroups form precipitin bands with spurs, indicating partial identity with the homologous strain (S. H. De Boer, *unpublished data*). It was not surprising, therefore, that strains in *E. c.* subsp. *atroseptica* serogroup XXII cross-reacted with the monoclonals to serogroup I. Evidently serogroup XXII strains have an O-side chain similar to that of serogroup I strains, but the weak reaction on the blot (Fig. 2) suggests either that the determinant was present at lower concentrations or that it has a lower affinity for the monoclonal than serogroup I strains.

Polyclonal antisera, in addition to the precipitin band reactions in immunodiffusion, react in ELISA and immunofluorescence with unknown antigens of whole cells that are not observed in immunodiffusion. The specificity of monoclonals with whole bacterial cells, on the other hand, is identical regardless of the serological procedure used, since they react with only a single antigen. Thus, the confusing picture that arises with polyclonal antisera in the various serological tests does not occur with the monoclonal antibody.

Monoclonal 4F6 could be used in the enzyme conjugate but not for coating plates in the ELISA procedure. Its reaction with the homologous bacteria, however, was appreciably weaker than ELISA with polyclonal antisera (Fig. 3). ELISA values with polyclonal antisera are high compared with those with monoclonal, probably because the monoclonal reacts with a single epitope, whereas the polyclonal antisera react with a large number of antigenic determinants. Background values observed with low dilutions of sap from healthy plants may have been caused by symptomless contamination of the stems with *E. c.* subsp. *atroseptica* or by nonspecific trapping of monoclonal-enzyme conjugate. Nonspecific trapping could perhaps have been reduced by more extensive washing of plates after antigen loading or by including a more extended blocking period.

Under antigen-limiting conditions, which presumably occur in tests of symptomless tubers contaminated by *E. c.* subsp. *atroseptica*, the number of epitopes that react is critical to the sensitivity of the assay. It is possible that the antigens expressed by metabolically inactive bacteria on tuber surfaces may be quantitatively or qualitatively different from those of actively growing cells, and this may account for the poor reaction of the monoclonal with tuber samples. The results suggest that the amount of LPS antigen available to react in ELISA is limited to a greater extent in the tuber samples than in pure cultures or active disease infections. Perhaps monoclonals to other antigens would be more useful in ELISA tests for detecting tuber contamination.

Results from the tuber tests with the monoclonal also may have differed from results with the polyclonal antisera because some of the polyclonal reaction may have resulted from cross-reaction or nonspecific antibody adsorption. Nonserological methods to establish independently the actual *E. c.* subsp. *atroseptica* content of naturally contaminated potato tubers are too insensitive to provide adequate controls. Moreover, artificially contaminated tubers probably do not provide a situation representative of natural contamination since relative antigen concentration could vary greatly between cells grown on laboratory media and those existing in a dormant state in potato periderm. In preliminary ELISA experiments with artificially contaminated tubers, ELISA values with monoclonal antibodies almost equaled polyclonal ELISA values when enzyme conjugate concentrations were adjusted to give about the same readings with pure cultures (S. H. De Boer, *unpublished data*). Further research will be required to establish whether a monoclonal or a mixture of monoclonals useful for detecting tuber contamination by *E. c.* subsp. *atroseptica* can be obtained.

TABLE 2. Comparison of monoclonal and polyclonal antibody-enzyme conjugates in enzyme-linked immunosorbent assay for detecting *Erwinia carotovora* subsp. *atroseptica* on potato tubers

Description[a]	No. of tubers	Absorbance at 405 nm			
		Monoclonal		Polyclonal	
		Mean	Range	Mean	Range
Tubers from blackleg-infected plants	8	0.09	0.05-0.13	0.46	0.23-0.70
Tubers from blackleg-infected plants	8	0.07	0.05-0.11	0.42	0.17-0.89
Tubers from ostensibly healthy plants	8	0.05	0.04-0.08	0.05	0.04-0.05
Tubers from ostensibly healthy plants	8	0.05	0.04-0.06	0.05	0.04-0.05
Random commercial tubers	16	0.85	0.37-1.66	>2.0	1.34->2.00
Random commercial tubers	48	0.21	0.15-0.32	0.81	0.22->2.00

[a] Tubers in each group were collected from a single field or storage bin. Undiluted tuber peel sap was used directly in the test.

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