Evaluation of Serological Tests for Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in Composite Potato Stem and Tuber Samples

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ABSTRACT

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A procedure to index potato seed lots for bacterial ring rot was evaluated by testing composite samples of tissue from 200 potato stems or tubers, in blind experiments, for the presence of *Clavibacter michiganensis* subsp. *sepedonicus* by immunofluorescence and enzyme-linked immunosorbent assay (ELISA) in six laboratories. Different antibody preparations were used for the two serological tests. Tissue samples were from ring rot–free potato plants, but ring rot–infected tissue was added to 88 of the 385 composite samples to create authentic positive composites. One laboratory achieved 100% efficiency for detection of ring rot infections in stem samples with ELISA, but the overall sensitivity and specificity of ELISA were 97.7 and 90.1%, respectively, for stems and 93.3 and 86.7%, respectively, for tubers. Efficiency of detection by immunofluorescence was 100% in three laboratories for stem and tuber samples. Overall sensitivity and specificity for immunofluorescence were 90.9 and 98.5%, respectively, for stems and 97.3 and 97.8%, respectively, for tubers. When a positive result in both serological tests was set as the requirement to designate a sample as infected with ring rot, specificity was 100% for stem samples in all laboratories and for tuber samples in four of the six laboratories. When a positive result for only one of the two serological tests was set as the requirement for designating a ring rot infection, sensitivity was 100% in all laboratories for stems and in five of the six laboratories for tubers, but specificity was reduced.

Serological testing of potato for *Clavibacter michiganensis* subsp. *sepedonicus* (Spieck. and Kotth.) Davis et al (syn. = *Corynebacterium sepedonicum* (Spieck. and Kotth.) Skapt. and Burkh.), the causal agent of the bacterial ring rot disease, is increasingly being used in Canada and the European Community to eradicate and prevent spread and introduction of the bacterial ring rot disease (8,13,21). Because the disease sometimes remains latent or symptomless in plants during the growing season and in tubers after harvest, undetected infections serve as an inoculum source, spreading the pathogen to "clean" seed lots and to ring rot–free geographic regions. In most countries, *C. m. sepedonicus* is considered a quarantine organism (2), and a ring rot exclusion policy was considered to have a very favorable cost-benefit ratio in the United Kingdom (17). Seed potato certification programs in Canada, the United States, and countries in the European Community have a zero tolerance for the disease (16,20).

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In 1987 the Commission of the European Communities published a scheme for the detection and diagnosis of the ring rot bacterium in composite samples of potato tubers (3). This scheme involves removing and pooling tissue cores from the heel ends of 200 potato tubers to form a composite sample, extracting and concentrating a bacteria-containing fraction after macerating the cores, and testing the bacterial fraction by immunofluorescence. Samples in which fluorescing coryneform-like bacteria are observed must be submitted to an eggplant bioassay from which *C. m. sepedonicus* must be isolated and its identification verified to obtain a positive diagnosis. The scheme, however, has its limitations. Specificity of the immunofluorescence test is hindered by serological cross-reactivity of antisera with saprophytic bacteria (5,7), and although the number of cross-reactions can be significantly reduced by antiserum dilution (15) and use of specific monoclonal antibodies (10), they remain a problem. Furthermore, inoculation of concentrated bacterial extracts from potato into eggplant is laborious and time-consuming and requires a considerable amount of greenhouse space. A minimum of 25 eggplants per sample is required for the bioassay in the European scheme. The sensitivity of eggplants to infection by *C. m. sepedonicus* is affected by the growing conditions of the eggplants, and erratic results may be obtained at low inoculum density (1,12,24). Moreover, eggplants, like potatoes, are liable to develop latent infections with *C. m. sepedonicus*. Symptoms are also obscured by the presence of other microorganisms, and certain other bacterial and fungal pathogens cause ring rot-like symptoms in eggplant (4,18).

Serological tests have already been used quite extensively for detection of ring rot in composite samples of potato tissue, but the efficacy of such testing has not been statistically analyzed. In this collaborative study we determined the sensitivity, specificity, and efficiency of the immunofluorescence test using antibodies to a somatic antigen and an enzyme-linked immunosorbent assay (ELISA) with a monoclonal antibody to an extracellular polysaccharide of *C. m. sepedonicus*. We considered application of the two serological tests independently and in combination. Furthermore, we compared efficiency parameters of the two serological tests as applied to composite samples of potato stem and tuber tissues. Although it is the tubers that are usually indexed for ring rot, testing of stems prior to harvesting of the crop has some advantages. Stems can be tested earlier than tubers, which enhances the usefulness of the results for grower management decisions. It is also often simpler to obtain random stem samples from the field than to obtain random tuber samples from a storage bin. Moreover, ring rot was detected with greater frequency in stems than in tubers in composite samples from field plots with known incidence of the disease (S. H. De Boer, *unpublished*).

MATERIALS AND METHODS

Composite samples. Composite samples of potato stems consisted of 200 1-cm segments taken near the base of stems of healthy plants and were provided by two Canadian and three European laboratories participating in this project. One or two stem sections from a ring rot–infected plant were included

in some of the composite samples supplied to each of the participating laboratories and identified by a code known solely to the supplying laboratory. Thirty composite stem samples, six to eight of which contained ring rot–infected tissue, were supplied to and tested in each of six laboratories (one shipment of five composite samples to one laboratory became lost in transit for many weeks and was not tested).

Composite tuber samples consisted of 200 cone-shaped, 0.5-g tissue plugs removed from healthy tubers at the stolon attachment site. As with the stem samples, tissue from ring rot–infected tubers was added to some of the tuber samples. Thirty-five composite tuber samples, of which 12–13 contained ring rot–infected tissue, were supplied to and tested in each of six laboratories.

Sample preparation. Composite samples of stem and tuber tissue were essentially processed according to the procedure of the European scheme (3); the process is shown in Figure 1. Different options were utilized in each of the testing laboratories for some of the processing steps (Table 1).

ELISA. ELISA tests were carried out on the filtrate or supernatant fraction of the first separation step depending on whether filtration or centrifugation was used. Microtiter plates were coated with one of several polyclonal antisera produced against whole cells in carbonate coating buffer (pH 9.6) overnight at 4 C or for 2 hr at 37 C. After washing out excess coating antibody, 50 µl of sample buffer (0.01 M phosphate buffer [pH 7.2] containing 2% [w/v] polyvinylpyrrolidone, 2% [v/v] Blotto, and 0.5 µl/ml Tween 20) were pipetted into each well, to which 50-µl aliquots of sample fluid were subsequently added. Samples were incubated in the plates at 4 C overnight and then washed from the wells. Nonspecific adsorption was blocked by incubating 2% Blotto in the wells for 30 min at 37 C. Subsequently, monoclonal antibody 1H3, anti-mouse immunoglobulin antibodies conjugated with alkaline phosphatase, and *p*-nitrophenyl phosphate substrate were added sequentially, washing the plates between each step, with 1-hr incubations at 37 C according to standard protocols (9). Pure cultures of *C. m. sepedonicus* at $A_{660} = 0.5$ were used as the positive control and extracts from healthy stem and tuber tissue were used for the negative control on each plate. Absorbance at 405 nm was measured when color development was clearly visible in positive control wells, which was usually after 1 hr at 37 C.

Immunofluorescence. Pellets from the final centrifugation step were suspended in distilled water, phosphate buffer, or phosphate-buffered saline (Table 2). Undiluted and three 10-fold dilutions of pellets were either heat- or acetone-fixed to multiwell microscope slides. They were stained by a standard indirect immunofluorescence staining procedure (10) with monoclonal antibody 9A1 in all but one laboratory, in which a polyclonal antibody was used. Preparations were observed by fluorescent microscopy at magnifications of 500–1,000×.

Test parameters. Sensitivity, specificity, and efficiency of serological tests were calculated on the basis of number of true and false positive and negative results obtained in each of six laboratories according to the formulas given previously (8). Sensitivity was defined as the percentage of actual positive samples detected by the test, i.e., when sensitivity is 100% there are no false negative test results. Specificity was defined as the percentage of actual negative samples identified correctly, i.e., when specificity is 100% there are no false positive results. Efficiency was defined as the total percentage of correct test results. These parameters were also calculated separately for combined results from all the laboratories.

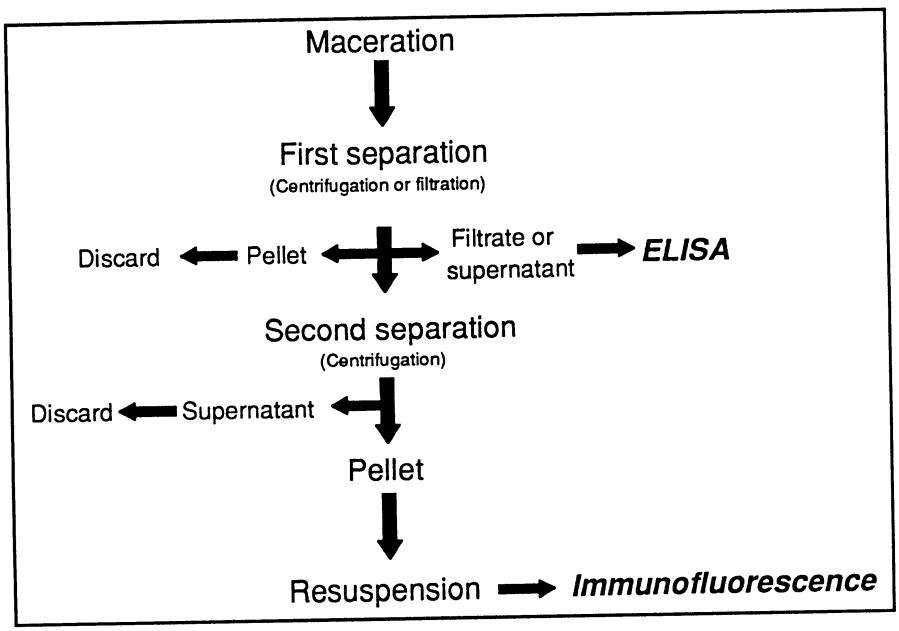

Fig. 1. Flow diagram for preparation of composite potato stem and tuber samples for serological tests.

RESULTS AND DISCUSSION

Efficiency of ELISA. Only one of the six laboratories achieved 100% efficiency in the ELISA test on composite stem samples and none achieved this with tuber samples (Table 2). The overall efficiency was similar for stem and tuber

Table 1. Options used in participating laboratories to prepare composite potato stem and tuber tissue samples for serological tests

Procedural step	Laboratory					
	A	B	C	D	E	F
Sample diluent	Distilled water	Distilled water	Distilled water	Maceration buffer	Maceration buffer	Distilled water
Diluent volume	1:2, w/v	1:1, w/v	1:1, w/v	1:2, w/v	25 ml	1:1, w/v
Homogenization	Blender	Blender	Blender	Blender	Bench vice	Blender
Settling time	None	None	None	30 min	None	None
First separation	Filtration Whatman No. 1	Centrifugation 190 *g*, 5 min	Centrifugation 190 *g*, 5 min	Filtration Whatman No. 1	Filtration glass filter G2 Whatman No. 3	Filtration Whatman No. 1
Second separation	Centrifugation 4,000 *g*, 20 min	Centrifugation 5,000 *g*, 20 min	Centrifugation 5,000 *g*, 20 min	Centrifugation 10,000 *g*, 10 min	Centrifugation 10,000 *g*, 20 min	Centrifugation 4,500 *g*, 15 min
Pellet diluent	Distilled water	Phosphate-buffered saline	Phosphate-buffered saline	Phosphate-buffered saline	0.01 M phosphate	Distilled water
Pellet concentration[a]	100-fold	4-fold	8-fold	200-fold	12.5-fold	150-fold

[a] Degree to which the bacteria-containing fraction was concentrated by centrifugation and resuspension calculated as the volume of liquid centrifuged per volume used to resuspend the pellet.

samples, however. Sensitivity of ELISA was 100% for stems in all but one of the laboratories; laboratory C had a single false negative test. Sensitivity was <100% in four of the six laboratories for tubers because of one or two false negative tests. Occurrence of false negative results was associated with suboptimal ELISA tests in which signal-to-noise ratios were low due to either relatively low absorbance (<0.600) in positive control wells or unusually high absorbance (>0.100) in negative control wells. Variation in ELISA tests appeared to be largely due to quality of the coating polyclonal antibody. Polyclonal antisera raised to whole fixed cells varied widely in effectiveness for coating (J. Mawhinney and S. H. De Boer, *unpublished*). Furthermore, batches of polyclonal antisera that initially gave good results in ELISA sometimes diminished drastically in effectiveness upon storage for several months at 4 C (J. Van Vaerenbergh, *unpublished*).

Specificity of ELISA was lower than sensitivity in most laboratories (Table 2). Only laboratories B and C attained 100% specificity for the stem samples, whereas other laboratories had false positive results for two to six stem samples and two to five tuber samples. Low specificity was probably the result of nonspecific adsorption or cross-reactions of antibodies with plant debris, soil mineral components, or saprophytic bacteria. The greater efficiency achieved with stem samples than with tuber samples is probably the result of more saprophytic bacteria and soil being associated with tubers than with stems. To some extent, the number of false positive as well as false negative reactions was a function of the threshold level. All the laboratories in our study used the same threshold levels, although the number of false test results could probably have been reduced by each using a different threshold level.

Efficiency of immunofluorescence. Overall efficiency of immunofluorescence was greater than that of ELISA for both stem and tuber composite samples (Table 2). The greater efficiency was due to an overall greater specificity, i.e., there were fewer false positive reactions with immunofluorescence than with ELISA. Greater specificity was attained with immunofluorescence than with ELISA probably because only cross-reactions with saprophytic bacteria are a problem in immunofluorescence, in contrast to ELISA, in which soil mineral components and plant debris may contribute to the problem. Moreover, cross-reactions with saprophytic bacteria in immunofluorescence often can be distinguished on the basis of bacterial cell morphology and intensity of staining.

Efficiency of the test on stem samples was 100% for three of the six laboratories, whereas in the other laboratories, one or two composite samples gave a false negative and/or false positive result. Similarly, three laboratories achieved 100% efficiency for tuber samples, whereas one of the other laboratories had no false positive results and another had no false negative results.

Parallel and serial tests. Specificity can be enhanced by the use of two serological tests utilizing antibodies that react with two different antigens of the target bacterium. In almost all cases, samples that gave a false positive or false negative reaction in one serological test did not do so in the other test. Thus, when test results were analyzed in a parallel fashion so that a positive test result in both ELISA and immunofluorescence was the prerequisite for judging a composite sample as a true positive, specificity of the test on stems increased to 100% for all laboratories (Table 3). An increase in specificity was also obtained for tuber samples but was less than 100% (95.5 and 95.8%) for two of the laboratories. Conversely, by setting the requirements for a true positive diagnosis to either a positive ELISA or a positive immunofluorescence test in serial fashion, sensitivity could be maximized; it was 100% for stems in all laboratories and for tubers in five of six laboratories (Table 4).

In practice, results from two serological tests could be used in parallel to obtain maximum specificity and in series to obtain maximum sensitivity. High specificity is especially important when testing domestic seed lots to ensure that growers do not unjustly suffer economic loss due to false positive diagnoses. On the other hand, sensitivity needs to be high for indexing seed lots destined for export in order to minimize the possibility of introducing the disease to ring rot-free areas. Laboratory testing, of course, does not obviate the need for fulfilling Koch's postulates to prove the presence of the ring rot bacterium in potato lots from geographic areas where ring rot is not endemic.

The high level of sensitivity and specificity achieved by combining results from six laboratories using incompletely standardized test protocols suggests that the combination of immunofluorescence and ELISA tests is an effective approach to testing composite samples for bacterial ring rot. With further standardization of protocols and antibody preparations, higher efficiency can probably be achieved.

Threshold values. The detection limit of ELISA for *C. m. sepedonicus* has been reported to be in the range of 1×10^5 to 5×10^6 cells per milliliter (6,23,25). Gudmestad et al (11) reported an effi-

Table 2. Sensitivity (Sens.), specificity (Spec.), and efficiency (Eff.) of ELISA and immunofluorescence tests for detecting *Clavibacter michiganesis* subsp. *sepedonicus* in composite samples of potato stems and tubers

	Stems			Tubers		
Laboratory	Sens.	Spec.	Eff.	Sens.	Spec.	Eff.
ELISA						
A	100.0	94.7	96.0	92.3	90.9	91.4
B	100.0	100.0	100.0	100.0	91.3	93.3
C	87.5	100.0	96.7	92.3	86.4	88.5
D	100.0	91.3	93.3	100.0	90.9	94.3
E	100.0	81.8	86.7	83.3	78.3	80.0
F	100.0	72.7	80.0	91.7	82.6	85.7
All	97.7	90.1	92.0	93.3	86.7	89.0
Immunofluorescence						
A	100.0	100.0	100.0	100.0	100.0	100.0
B	100.0	100.0	100.0	100.0	100.0	100.0
C	85.7	100.0	96.7	100.0	100.0	100.0
D	71.4	91.3	86.7	100.0	90.9	94.3
E	100.0	100.0	100.0	83.3	100.0	94.3
F	87.5	100.0	96.7	100.0	95.7	97.1
All	90.9	98.5	96.6	97.3	97.8	97.6

Table 3. Sensitivity (Sens.), specificity (Spec.), and efficiency (Eff.) of serological tests for detecting *Clavibacter michiganesis* subsp. *sepedonicus* in composite samples of potato stems and tubers when results of both ELISA and immunofluorescence must be positive to consider the composite sample positive

	Stems			Tubers		
Laboratory	Sens.	Spec.	Eff.	Sens.	Spec.	Eff.
A	100.0	100.0	100.0	100.0	100.0	100.0
B	100.0	100.0	100.0	100.0	100.0	100.0
C	71.4	100.0	93.3	100.0	100.0	100.0
D	71.4	100.0	93.3	100.0	95.5	97.1
E	100.0	100.0	100.0	83.3	100.0	94.3
F	87.5	100.0	96.7	100.0	95.8	97.1
All	88.6	100.0	97.1	97.2	98.6	98.1

ciency of 94% at populations of target bacteria $>10^3$ cfu/g of plant tissue. Threshold values for ELISA are often set by various calculations such as adding two, three, or four times the standard deviation to a mean negative control value or by simply using two or three times the negative control value itself (22). No method, however, seems to be universally acceptable. When negative control values are very low (e.g., <0.01) and approach zero, threshold values cannot sensibly be based on the negative control because their 2–4× multiples and standard deviations fall below values that can occur sporadically with negative field samples due to test variability. Field samples, such as crude plant extracts, may contain various factors, including phosphatase and nonspecific protein-binding compounds, that affect background color development in ELISA. In our study, therefore, the ELISA threshold was set at 0.100 for tests in which the mean absorbance of negative control wells was ≤0.060. When absorbance of negative controls exceeded this value, the threshold was put at three times the mean of the negative controls. Using these thresholds, 8.3% of the 385 composite samples tested gave a false positive response but only 1.6% gave a false negative reading.

The degree to which the supernatants or filtrates were concentrated by centrifugation to prepare suspensions for immunofluorescence varied from fourfold to 200-fold among laboratories (Table 1). Furthermore, the volume of sample applied to slides, diameter of wells on slides, and microscope magnification varied among laboratories. Nevertheless, most of the positive samples had more than 50 cells per microscope field in at least one of the dilutions, whereas negative samples usually had fewer than one cell per field. No cells were observed in 84% of the negative samples from all laboratories. Although it is recognized that the presence of any fluorescing cells potentially indicates the presence of ring rot bacteria, the occasional presence of cross-reacting bacteria necessitated setting a threshold level, as pointed out by Samson et al (19). A threshold of one cell per field was used by Gudmestad et al (11) for individual plant samples, but a higher threshold is required for composite samples from which a bacteria-enriched fraction is extracted and concentrated. Miller (14,15) variously reported background levels of cross-reacting bacteria at 2.5×10^3 and 2×10^4 cells per milliliter in concentrated extracts of composite tuber samples. In the Canadian diagnostic laboratories, the threshold has been set at five cells per field because counts of fluorescing bacteria in almost all samples are well above or below this value. Samples with more than five cells per field almost invariably also give positive latex agglutination and ELISA readings, and samples with fewer than five cells per field are negative in other serological tests. In this study, the logarithm of the product of the mean number of cells per field and the dilution factor were calculated and the threshold was set at 2.7. Because of differences in procedures and in slide and microscope configurations among laboratories, this threshold was not interpreted in terms of actual density of bacterial cells in the sample extract but merely served as a useful benchmark to delineate positive from negative samples. With this threshold, only 1.6 and 1.8% of the composite samples gave false positive and false negative readings, respectively.

The necessity of setting threshold levels introduces the problem of samples with test results near the threshold level. Potato consignments with test results in the "gray area" near the threshold cannot be unequivocally diagnosed. Fortunately, in practice very few test results are in the gray area. For example, only 0.17% of about 12,000 seed lots tested in the Seed Potato Testing Laboratories in Charlottetown and Fredericton during the last 4 yr gave equivocal immunofluorescence test results (T. L. De Haan and J. Mawhinney, *unpublished*).

Probability of detection. Our results are based on composite samples to which were added one or two tissue segments from known infected plants containing high populations of ring rot bacteria. Previous work with single plants showed that ring rot detection at low inoculum levels was significantly less than at high inoculum levels (9). It would, therefore, be anticipated that sensitivity and specificity are also functions of the concentration of target bacteria in the sample. In the indexing procedure, bacterial fractions were concentrated fourfold to 200-fold for immunofluorescence (Table 1), but soluble antigen was tested on unconcentrated samples in ELISA. This may account, in part, for differences in test sensitivity. Nevertheless, serological tests for a single bacterial species in a complex milieu like plant samples obviously cannot be used to detect as few as one cell per sample. The actual number of cells that can be detected by immunofluorescence with confidence depends not only on antibody specificity but also on how well the bacteria can be extracted and separated from the plant tissue. The ELISA test is particularly dependent on the signal-to-noise ratio of the system being used. Finally, the probability of detecting bacterial ring rot in a consignment of potatoes depends on sample size, how well the sample represents the consignment, and the incidence of infection even when sensitivity and specificity of the laboratory test are definitive.

Table 4. Sensitivity (Sens.), specificity (Spec.), and efficiency (Eff.) of serological tests for detecting *Clavibacter michiganesis* subsp. *sepedonicus* in composite samples of potato stems and tubers when results of either ELISA or immunofluorescence must be positive to consider the composite sample positive

	Stems			Tubers		
Laboratory	Sens.	Spec.	Eff.	Sens.	Spec.	Eff.
A	100.0	94.7	96.0	100.0	90.9	94.3
B	100.0	100.0	100.0	100.0	91.3	94.3
C	100.0	100.0	100.0	100.0	86.4	91.4
D	100.0	87.6	86.7	100.0	86.4	94.1
E	100.0	81.8	86.7	83.3	78.3	80.0
F	100.0	72.7	80.0	100.0	79.2	85.7
All	100.0	88.5	91.4	97.3	85.3	89.5

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