Proficiency Testing in a Laboratory Accreditation Program for the Bacterial Ring Rot Pathogen of Potato

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ABSTRACT

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Variability of enzyme-linked immunosorbent assay (ELISA) and immunofluorescence tests for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato tissue was analyzed to determine the magnitude of repeatability (within analyst variation) and reproducibility (among analyst variation) components. The analysis was based on data generated by analysts in eight laboratories testing proficiency panel samples distributed under a laboratory accreditation program. The standard deviation for repeatability of the ELISA test was small but increased at higher absorbance readings, while the standard deviation for reproducibility was larger and also increased at high absorbances. For immunofluorescence, the standard deviation for repeatability and reproducibility were similar to one another and increased with increasing bacterial concentration, as might be expected for count data and the inherent subjectivity of the test. The reproducibility standard deviation provided the basis for calculating "z-scores" by the Association of Official Analytical Chemists' procedure to evaluate proficiency of chemical analyses. More than 90 and 80% of the z-scores for samples tested in this study by ELISA and immunofluorescence, respectively, were in the acceptable range. The rescaled sums of z-scores for individual analysts were used as single combination scores to evaluate each analyst's results over all samples of a proficiency panel. This measure may be useful for tracking analyst performance on process control charts as part of a quality control system.

Ring rot disease of potato, caused by the gram-positive, coryneform bacterium, *Clavibacter michiganensis* subsp. *sepedonicus,* is largely controlled by the use of disease-free seed potatoes. Although the disease devastated potato crops in Canada during the 1940s and 1950s, it only occurred sporadically during the 1970s and 1980s as a result of a zero tolerance for the disease in all classes of seed. Visual field inspections of growing crops and harvested tubers served to identify infected lots that needed to be removed from the seed certification program. The survival of the bacterium in symptomless crops, however, resulted in a failure to eradicate the disease (8). The disease persisted and occasionally caused serious economic losses to individual growers, largely because of the zero tolerance rule, and became a major obstacle to international trade of Canadian seed potatoes.

In 1979 and the following years, laboratory testing to detect the possible presence of *C. michiganensis* subsp. *sepedonicus* in seed lots that had passed field inspection was initiated in Canada to facilitate international trade. By 1985, the advantage of laboratory testing for detecting incipient ring rot infections had become clear and testing of domestic seed lots was introduced on a voluntary basis in some provinces. By 1992, the specificity of laboratory testing had improved significantly through the introduction of monoclonal antibodies for both the enzyme-linked immunosorbent assay (ELISA) and immunofluorescence tests, and laboratory indexing of all seed potatoes for *C. michiganensis* subsp. *sepedonicus* in Canada became mandatory. To minimize cost, testing is done in serial fashion by initially screening seed lots using ELISA and then testing all ELISA-positive lots by immunofluorescence for confirmation. A positive test in both ELISA and immunofluorescence, indicating the presence of both *C. michiganensis* subsp. *sepedonicus*-specific extracellular polysaccharide (EPS) and somatic antigen epitopes (6), is required for a sample to be considered infected with *C. michiganensis* subsp. *sepedonicus*.

With privatization of potato testing in Canada, an accreditation program was implemented to ensure that reliable and uniform results were obtained from multiple laboratories. Hence the test program for *C. michiganensis* subsp. *sepedonicus* became one of the first to apply a specific international quality assurance standard to laboratory detection of a plant pathogen. Today, the quality assurance program of each private laboratory must follow the criteria set by the International Standards Organization (ISO) in their guide 25 (1). In addition, testing for *C. michiganensis* subsp. *sepedonicus* under the accreditation program requires the use of validated antibody sources as well as standardized positive and negative controls, and involves on-site audits and training. Analysts in private laboratories are required to complete correctly blind "proficiency panel" samples on a semiannual basis to maintain their certified status, which allows them to conduct the tests in an accredited laboratory. These proficiency tests are administered by the Centre of Expertise for Potato Diseases of the Canadian Food Inspection Agency. Although analysts need only to identify each sample correctly as positive or negative to maintain their certified status, they do provide actual absorbance readings from the ELISA tests and counts of the number of coryneform-like fluorescing bacterial cells per microscope field from the immunofluorescence tests. In this study, we used these quantitative data to evaluate the proficiency of the analysts more quantitatively.

Protocols for assessing proficiency in doing chemical analyses have been developed by the Association of Official Analytical Chemists (AOAC; 13), and have also been used to assess the proficiency of analytical laboratories testing foods and feeds (11,14). Although serological tests for plant pathogens generally have not been conducted at the level of quality control used for official chemical analyses, the same principles do apply. After all, the monoclonal antibody used in ELISA targets a specific molecular moiety whose concentration ultimately determines the level of substrate conversion to a colorogenic product. Use of the AOAC protocol for proficiency testing requires some knowledge of the interlaboratory variability encountered with the analytical procedure. This knowledge can be obtained through method-performance (collaborative) studies and a protocol is available for this type of study (9). In our investigation, we determined the interlaboratory variability and then used the AOAC statistical approach to analyze the results from both the ELISA and immunofluorescence profi-

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ciency panels for *C. michiganensis* subsp. *sepedonicus*.

MATERIALS AND METHODS

Participating laboratories. The Centre of Expertise for Potato Diseases (CEPD) in Charlottetown, PE, Canada served as the reference laboratory. Eight private laboratories located across Canada met all the quality assurance requirements to be accredited to conduct tests for *C. michiganensis* subsp. *sepedonicus* by the Canadian Food Inspection Agency. Up to 17 certified technical analysts conducting the laboratory work were trained at CEPD, used official protocols, and worked in laboratories with an approved and audited quality assurance system. In each participating laboratory, one to four analysts, working independently, completed the proficiency panels.

Test protocols. The ELISA and immunofluorescence procedures used in the tests have been briefly described elsewhere (4) and are given in detail in the official protocol (2). The ELISA test for each sample was conducted in duplicate wells of 96-well microtiter plates precoated with anti-*C. michiganensis* subsp. *sepedonicus* rabbit polyclonal antibody, and monoclonal antibody 1H3 was used as the specific probe for *C. michiganensis* subsp. *sepedonicus*. ELISA plates were developed with p-nitrophenyl phosphate substrate as the signal molecule for the alkaline phosphatase-conjugated second antibody in a triple-antibody sandwich ELISA format. Positive control samples, consisting of lyophilized extracellular polysaccharide antigen harvested from agar plate cultures of *C. michiganensis* subsp. *sepedonicus* and diluted to give ELISA absorbance readings of 0.8 to 1.2 at 405 nm, were provided to each accredited laboratory by CEPD. Negative control samples, consisting of lyophilized healthy tuber extract giving ELISA absorbance readings of 0.001 to 0.050, were similarly provided. In accordance with the official test protocol, positive and negative controls were each loaded in quadruplicate in rows 1 and 12 of each microtiter ELISA plate. Samples were duplicated on the left side (rows 2 to 6) and the right side (rows 7 to 11) of a plate. The absorbance value for each sample was transformed by $x = (X - Y_n)/(Y_p - Y_n)$, where x is the transformed value, X is the actual absorbance reading, Y_n is the mean of the four negative control values on the same side of the plate as the sample, and Y_p is the mean of the four positive control values on the same side of the plate as the sample.

Bacteria-enriched fractions from tuber extracts were prepared for immunofluorescence by the differential centrifugation procedure (7), also as detailed in the official protocol (2). Fifteen μl of 1:10, 1:50, and 1:100 dilutions of the final suspension were acetone-fixed to 4-mm windows of glass microscope slides. Immunofluorescence was carried out on the fixed preparations using monoclonal antibody 9A1 with a fluorescein conjugate by the indirect staining procedure as described (4). The number of fluorescing coryneform-like bacteria at 1,000× magnification in 30 microscope fields was recorded as the average number of immunofluorescing units (ifu) per field. Duplicate readings were not taken for the immunofluorescence test and only data from the 1:50 dilutions were analyzed in this study.

Proficiency samples. The samples used for the proficiency tests for ELISA consisted of different potato extracts from either healthy or ring rot-infected potato tubers. For the purposes of this study, only the results from the positive extracts were used. Two sets of samples were prepared and tested at several different times by each participating laboratory. Set A was used in the proficiency panel tested by the laboratories in September 1996 and April 1997, and consisted of 24 extracts, 16 of which were from positive ring rot-infected tubers. Set B was used in the proficiency panel for December 1997, May 1998, and October 1998, and consisted of 17 extracts, 8 of which were from ring rot-infected tubers. Each positive sample in set A was from a separate extract but all were strongly positive, giving high absorbance values (0.7 to 1.3) in the ELISA test. The positive samples from set B consisted of two blind replicates of each sample and were selected to give both low and high absorbance values (0.06 to 1.3). The ELISA test for each sample, including blind replicate samples, was performed in duplicate on each test date.

As with ELISA, two sets of immunofluorescence samples were used. The cell count ranged from 5 to 90 ifu/field in set A and from 5 to 25 ifu/field in set B. In set A, there were nine positive and three negative samples, whereas in set B there were eight positive and four negative samples. The dates of the immunofluorescence panels were the same as those for ELISA.

The true or assigned value sensu Thompson and Wood (13) for each ELISA and immunofluorescence sample for Set B was the mean of 20 determinations, 10 of which were made by each of two analysts in the reference laboratory over a 5-week period. Such data were not available for set A and, therefore, the consensus value which is the average of the values from the participating analysts was used as the assigned value for each sample in this set.

Statistical analysis: ELISA. The proficiency panel conducted in December 1997 (set B) was used as a method-performance study (9) to estimate the experimental variability of the analytic ELISA method. The protocol refers to variability among and within laboratories but, for our purposes, it was more appropriate to focus on analysts rather than laboratories. It is the proficiency of the analyst that is of interest.

Following the protocol, outliers in the data set were identified by Cochran's and Grubbs' tests. For this purpose, the values for the duplicate determinations were averaged. The data set without outliers was used in subsequent calculations. Variance components were estimated by relative maximum likelihood using SAS statistical software (SAS Institute, Inc., Cary, NC). Initially, the components of variance that were estimated were analysts, replicate samples within analysts, and duplicates within replicates. The variance component for the replicates was zero for five of the eight samples, which suggested that the variability among replicates was no greater than the variability among laboratory duplicates. Consequently, the data were reanalyzed with only two sources of variation: analysts and within analysts. The within-analyst component included the variability between replicate samples and between duplicate determinations. The log (base 10) of the square root of each variance component was regressed on the assigned values to determine whether they were related (3,12). If they were not, the average of the variance component was calculated. The results were used to calculate the repeatability (i.e., within analysts) and reproducibility (i.e., among analysts) standard deviations.

To assess the proficiency of the analysts, the ELISA data from the remaining panels were evaluated using the protocol for proficiency testing (13). The result of each sample for each analyst was assessed by calculating a "z-score" value. The z-score is $z = (x - \hat{x})/\sigma$, where x is the result obtained by the testing laboratory (the transformed ELISA value), and $\hat{x}$ is the assigned (or true) value for the sample. The reproducibility standard deviation determined from the December 1997 data provided the basis for the target value (σ) for the standard deviation. The scores were calculated for each sample done by each analyst and were accumulated over all samples done by a single analyst to give the rescaled sum of scores, a single assessment of the analyst's work for each proficiency panel. The rescaled sum of scores is given by $\Sigma z/\sqrt{n}$, where n is the number of z-scores being combined (13). When the analyst's result agrees well with the assigned value, the z-score will be small. In practice, scores between –2 and +2 are considered acceptable, while those outside the range may indicate problems (11,13).

Statistical analysis: immunofluorescence. The immunofluorescence assay is more difficult and laborious to carry out than ELISA, so replicate samples were not provided to the analysts and immunofluorescence readings were not duplicated. Consequently, data from the December 1997 and May 1998 panels, which used the same samples, were used as replicates in

the method performance study. Using the data from the two panels in this way required the assumption that the assigned values for the samples did not change over the 6-month interval between administration of the panels. The methods used in screening for outliers, estimating variance components for analysts and within analysts, estimating relationships of variance components with the assigned values, and determining the repeatability and reproducibility standard deviations were the same as for the ELISA data.

To assess the proficiency of the analysts, the immunofluorescence data from the remaining panels were evaluated using the protocol for proficiency testing (13). Again, the proficiency panel results were assessed by calculating a z-score for each sample for each analyst and the rescaled sum of scores for each analyst as was done with the ELISA data.

RESULTS

ELISA. The ELISA data for the positive samples from the December 1997 panel were suitable for use as a method performance study. Cochran's and Grubbs' tests identified two ELISA values for one sample and one value each for three other samples as outliers. These values were eliminated from the data set used to estimate the variance components and standard deviations. The log of the variance component for within analysts was a function of the ELISA value ($R^2 = 0.89$ for all data, and 0.85 for data without outliers) but the variance component for between analysts was not. The estimate of the standard deviation for repeatability (S_r) was

$$S_r = 10^{(0.5656x - 1.5942)}$$

where x is the transformed absorbance value for the sample. The estimated standard deviation for reproducibility (S_R) was determined to be

$$S_R = \sqrt{0.01937 + 10^{(1.1312x - 3.1884)}}$$

Both the repeatability and reproducibility standard deviations were related to the absorbance (Fig. 1).

Immunofluorescence. The assumption that there were only random differences between the immunofluorescence data from December 1997 and May 1998 proficiency samples was tested by analysis of variance. The data for each sample were analyzed separately, with dates and analysts as factors. There were significant differences between dates for two of the samples but the values for one sample had increased from December to May and the other decreased. There was no obvious explanation for these differences and they were not consistent; therefore, these samples were included in the rest of the investigation.

Grubbs' and Cochran's tests identified four values for one sample and one value for each of two other samples as outliers. These values were eliminated from the data set used to estimate the variance components and standard deviations. The log of the variance component for within analysts was a function of the mean ifu count ($R^2 = 0.85$ for all data and 0.60 for data without outliers) but there was not a significant correlation between the log of the variance component for between analysts and ifu count. The estimated standard deviation for repeatability S_r was

$$S_r = 10^{(0.02844y + 0.1940)}$$

where y is the cell count as ifu per microscope field. The estimated standard deviation for reproducibility (S_R) was

$$S_R = \sqrt{0.9600 + 10^{(0.05688y + 0.3880)}}$$

The repeatability and reproducibility standard deviations change considerably over the range of immunofluorescence counts normally encountered in this test (Fig. 2).

Z-scores. We used the reproducibility standard deviation for calculating the value of the target standard deviation σ for determining the z-scores in the proficiency studies. The sample mean for each analyst is based on k determinations. Thus, for ELISA

$$\sigma = \sqrt{0.01937 + 10^{(1.1312x - 3.1884)} / k}$$

and for immunofluorescence,

$$\sigma = \sqrt{0.9600 + 10^{(0.05688y + 0.3880)} / k}$$

where x and y are the assigned absorbance value and ifu count for the sample, respectively. Samples were duplicated in ELISA so $k = 2$, but $k = 1$ for immunofluorescence samples which were not duplicated. For the proficiency panel samples, assigned ELISA absorbance values varied from 0.060 to 1.280, giving σ values of 0.1405 to 0.1687. Similarly, for immunofluorescence, assigned values varied from 5 to 25 ifu/field, giving σ values of 2.3798 to 8.0950. Except for the very first panel, conducted in September 1996, the z-scores obtained from the ELISA results for each panel were symmetrically distributed around zero with a small positive tail (Fig. 3). In that first panel, only 86.6% of the z-scores were in the acceptable range of –2 to +2, but 91.1, 99.0, and 91.8% of the z-scores were in the acceptable range for the panels conducted in April 1997, May 1998, and October 1998, respectively. The z-score distribution for immunofluorescence also was skewed toward higher values (Fig. 4). The percentage of z-scores for immunofluorescence in the acceptable range was 93.6, 82.5, 85.6, and 84.8 for the panels conducted during September 1996, April 1997, May 1998, and October 1998, respectively.
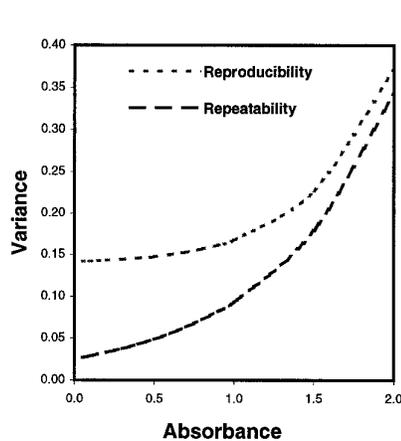

Fig. 1. Repeatability (---) and reproducibility (…) standard deviations within and among analysts, respectively, in an interlaboratory study for the range of absorbance values normally encountered in testing for the bacterial ring rot pathogen using a triple-sandwich antibody enzyme-linked immunosorbent assay format with monoclonal antibody 1H3.
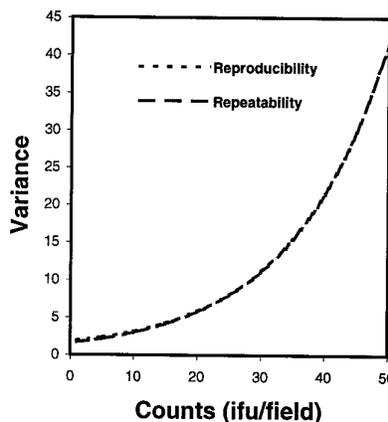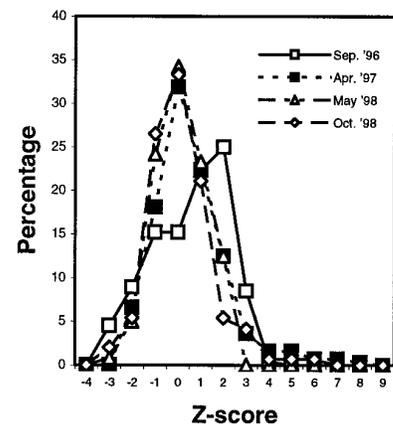

Fig. 2. Repeatability (---) and reproducibility (…) standard deviations (largely overlapping lines) within and among analysts, respectively, in an interlaboratory study for the range of cell counts, given as immunofluorscing units (ifu) per microscope field, normally encountered in testing for the bacterial ring rot pathogen using indirect immunofluorescence with monoclonal antibody 9A1.

Fig. 3. Distribution of z-score values for enzyme-linked immunosorbent assay results for samples tested by analysts completing proficiency testing in eight laboratories on four different dates.

The rescaled sums of z-scores for ELISA for the analysts that participated in October 1998 proficiency test ranged from –5 to 8 (Fig. 5). The rescaled sums for immunofluorescence were in the range of –2 to 4 (Fig. 6).

DISCUSSION

The current approach to ring rot testing in Canada, with its feasible and affordable laboratory test procedure, provided suitable data for a statistical evaluation of analyst proficiency. Although the data used for estimating the variance components and standard deviations were not derived from a method-performance study per se, the criteria for such a study were met in that all laboratories analyzed the same samples by the same methods (15).

The standard deviation for repeatability, which is the actual variation in results for repeated tests on the same sample by a single analyst, was small for the ELISA test, which attests to the precision of the method employed (Fig. 1). That the standard deviation for repeatability increased with the absorbance reading is not surprising and may be a function of the test itself, such as loss of some antigen during the washing procedure at high antigen concentration, rather than being a reflection of technical ability. The standard deviation for reproducibility was less dependent on the absorbance reading up to values of approximately 1.25 and reflects the differences in results that occur when different analysts test the same sample. This variation could be due to factors such as differences in the concentration of reagents, plate washing technique, and microplate readers. The level of consistency we observed in ELISA results attained among analysts is probably due, at least in part, to the practice of transforming the raw data on the basis of standardized positive and negative controls included on each microtiter plate.
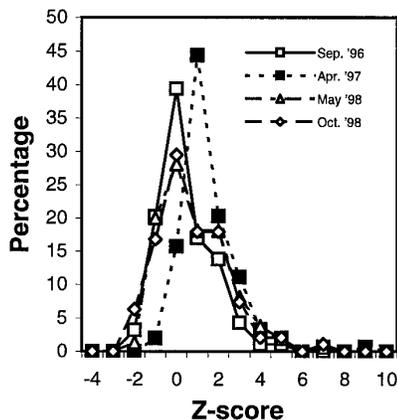
Horowitz et al. (10) described the universal increase in the coefficient of variation (CV) of analytical tests with decreasing concentration of the detection target due to the inherent greater inaccuracies in detecting very low concentrations. While we did not calculate the Horowitz CV function, the CV did increase as the absorbance reading in ELISA decreased (*data not shown*). This implies that results of laboratory analyses may have a greater percentage of deviation from the true value at the positive-negative threshold than at higher levels of infection. Nevertheless, previously set threshold criteria resulted in correct results for the proficiency samples. Increasingly more erroneous results might be expected, however, if thresholds were to be lowered with the need to detect smaller and smaller concentrations of assay target (i.e., bacterial EPS). Sensitivity and specificity of the test then become critical and, in fact, may be the limiting factor in obtaining correct results.

For immunofluorescence, repeatability and reproducibility increased quite dramatically with increasing bacterial concentration (Fig. 2). The large standard deviation at high cell numbers is natural in count data but may also be the result, in part, of the tedium of manually counting many cells and the inherent subjectivity in the test. In contrast to ELISA, which targets a soluble antigen, the immunofluorescence test targets whole bacterial cells and requires the analyst to visually identify and count fluorescing entities that meet size and shape criteria for coryneform bacterial cells. Moreover, the analysis we performed may have overestimated the standard errors because the data we used were from two proficiency panels rather than from replicate samples measured at the same time. The large variability in counts for samples with high bacterial populations, however, is unlikely to have a bearing on whether a sample is negative or positive for the presence of *C. michiganensis* subsp. *sepedonicus* because the positive-negative threshold is at only 5 ifu/field (4).

The z-scores provided a method for assessing the proficiency of analysts employed to determine the presence of *C.*
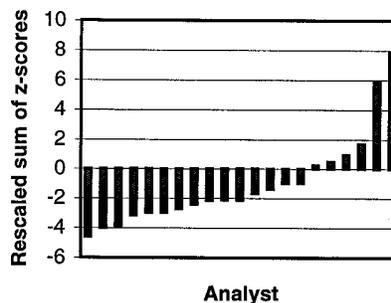
michiganensis subsp. *sepedonicus* in potato extracts. More than 90% of the z-scores from the ELISA test were in the acceptable –2 to +2 range and at least 80% of the immunofluorescence test z-scores were in this range (Fig. 3 and 4). The z-scores for the ELISA test show that results obtained by analysts in the accredited laboratories deviated little from those obtained in the reference laboratory. Most deviant z-scores were >2 and these were not of great concern in the context of the Canadian ring rot testing program because readings that are too high would tend to give false positive rather than false negative results. All samples that test positive for ring rot in the accredited laboratories are submitted to the reference laboratory for confirmation to ensure that a correct diagnosis is obtained. Such safeguards are necessary because of the enormous socioeconomic consequences of a positive ring rot diagnosis on seed potato farms (5). False negatives also have serious consequences because they result in persistence of the pathogen in the seed potato lot with concomitant risk of infecting contact lots and introduction of the pathogen to other geographic areas. However, the failure to detect the pathogen in a seed potato crop is more likely due to small sample sizes than to shortcomings of the laboratory test. The sample size is limited by practical considerations.

Several methods are available for combining the scores for each analyst over all samples from a proficiency panel (13). We used the sum of the z-scores rescaled according to the number of scores being combined. For ELISA, rescaled sums of z-scores both <–2 and >+2 were observed, suggesting consistent biases in analysts' results. Thus, some analysts tended to have more negative z-scores while others tended to have more positive z-scores than expected, even though z-scores for individual samples per analyst were largely within the acceptable range. The range of rescaled sums of z-scores for immunofluorescence was less than for ELISA. This is a reflection of the relatively high target standard deviation accepted for immunofluorescence minimizing z-score values. Overall, the combined scores for most analysts
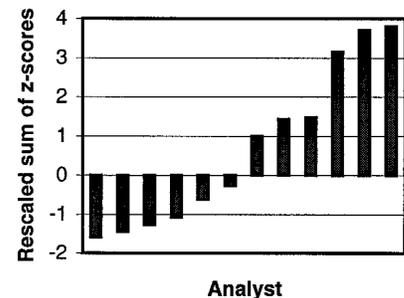

Fig. 4. Distribution of z-score values for immunofluorescence results for samples tested by analysts completing proficiency testing in eight laboratories on four different dates.

Fig. 5. Rescaled sum of z-scores for the enzyme-linked immunosorbent assay tests of each analyst participating in the proficiency test of October 1998.

Fig. 6. Rescaled sum of z-scores for the immunofluorescence tests of each analyst participating in the proficiency test of October 1998.

were within the acceptable range for immunofluorescence and all meaningful deviations were biased toward higher readings.

Although ultimately, with a zero-tolerance disease like bacterial ring rot, the actual level of pathogen density or concentration is not an issue because all infections result in the same regulatory action, it is reassuring to find that the tests give similar quantitative results in all laboratories. Furthermore, calculation of the rescaled sum of z-scores may be a useful measure for tracking analyst performance on process control charts as part of a quality control system.

ACKNOWLEDGMENTS

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