LABORATORY CONSISTENCY IN EXTRACTION OF NEMATODES FROM SOIL AND ROOTS

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ABSTRACT

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We found inconsistency in reports of extracted nmatodes between two nematode diagnostic laboratories. Each laboratory analyzed 200 matched soil and 200 matched root samples. Agreement in detection and nondetection for three nematode species in soil was less than 85%, and in roots agreement was greater than 85% for one of the three species. Relationships were not found to support consistency between matched samples in which the same nematode was detected by both laboratories. An explanation, in part, for the inconsistency may lie in the dilution and counting procedure of the extraction process. A first approximation to a model for minimizing counting errors suggests that, when dilution is required for the counting of nematodes, differing dilutions should be used, and that, if a single sample is taken, about 840 nematodes should be present in the sample. However, the same precision can be achieved with about 170 nematodes per sample if four samples are counted and averaged.

Additional key words: nematode diagnostic laboratories, phytoparasitic nematodes, quantitative nematology, quality control, sampling.

RESUMEN

Brown, S. M., M. F. Miller, y D. R. Viglierchio. 1987. Consistencia entre laboratorios en la extracción de nematodos del suelo y raíces. Nematropica 17:179-192.

Se encontró inconsistencia en los resultados de extracción de nematodos entre dos laboratorios de diagnóstico de nematodos. Cada laboratorio analizó 200 muestras similares de suelo y 200 raíces. Concordancia en la detección y no detección para tres especies de nematodos en el suelo fue menos del 85%, mientras que en las raíces, la concordancia fue mayor del 85% para una de las 3 especies. No se encontraron relaciones para sustentar la concordancia entre muestras similares, en las cuales, el mismo nematodo fue detectado por ambos laboratorios. Esta inconsistencia, podría estar relacionada, en parte, con la dilusión y conteo del proceso de extracción. La primera aproximación a un modelo para minimizar errores de conteo, sugiere que cuando se requiere una dilusión para el conteo de nematodos, dilusiones diferentes deben ser utilizadas, y que si una muestra es tomada, alrededor de 840 nematodos deberían estar presentes en la muestra. Sin embargo, la misma precisión puede ser lograda con alrededor de 170 nematodos por muestra si cuatro muestras se cuentan y promedian.

Palabras claves adicionales: laboratorios de diagnóstico de nematodos, nematodos fitoparásitos, nematología cuantitativa, control de calidad, muestreo.

INTRODUCTION

The effectiveness of alternative technologies in nematode control is predicated largely on quantitative nematology. Quantitative estimates of plant-parasitic nematodes in soil and plant tissue remain critical to both nematological research and control recommendations. To be of quantitative value, a method for extraction must provide an accurate estimation of the true number of nematodes present in a sample with a minimum of error. Despite decades of nematological research, no current method is able to accomplish this requirement faithfully. The more common practices are based upon any of several assumptions including: 1) the extraction process is complete (100% effective); 2) the extraction process is incomplete (efficiency not estimated), but because the method is applied to all samples, the results are related and therefore comparable; 3) a method used in one laboratory set of conditions can be applied directly in another laboratory elsewhere with no testing or calibration.

In an examination of several methods for extraction, Viglierchio and Schmitt (10) demonstrated that extraction efficiency of certain nematodes from soil was dependent upon method, soil type, and nematode species. They found that five common methods did not achieve a recovery of even half of the nematode inoculum and that the variation obtained among trials was too large for the use of correction factors. These problems are likely to be important in consideration of the varying results that have been reported from different laboratories (9) and must be dealt with before introducing procedures for quality control.

During the planning of a special project that required the estimation of nematode populations from samples of soil and roots collected over a large agricultural area with varying soil types and species of nematodes attacking bananas, our effectiveness in evaluating population estimates of nematodes was seriously handicapped by the absence of quality control procedures in the diagnostic laboratories. Fortunately we chose to submit matched samples to two laboratories for analysis of nematodes to obtain independent estimates (8).

Our purpose here is to demonstrate the fallacy in nonstandardized protocols without quality control by comparing the results of matched samples submitted to each laboratory. Each of 200 soil and 200 root samples was randomly divided in half by mounding and quartering, and one matched half was processed by each laboratory. The identity of the laboratories has been withheld to provide comparison without prejudice.

MATERIALS AND METHODS

Laboratory procedures. The Laboratory A normal practice extracted nematodes from soil and roots by sieving and centrifugation (7). Fifty-cm^3 samples of soil were mixed with approximately 2L of water and

decanted through stacked sieves (147 μm, 43 μm, and 43 μm apertures). The sievings from each 43 μm sieve were collected. This procedure was repeated once with the soil residue. The combined sievings were spun in a centrifuge; the supernatant was discarded, and the pellet suspended in a sugar solution (specific gravity 1.18). Centrifugation was repeated for 3 min, and the supernatant sieved with a 43 μm sieve. The sievings were rinsed into a counting dish, and the nematodes counted and reported as nematodes per 50 cm^3 of soil. For root analysis, 10 g of roots in water were chopped in a blender. The resulting mixture was poured through stacked sieves as described previously, and processed as with soil except that sugar supernatant sievings were collected in a volume of 40 ml of water. This suspension was mixed, and the nematodes in a 5-ml sample were counted. The counts were multiplied by 8 to report nematodes per 10 g of roots.

The Laboratory B normal practice extracted nematodes from soil and from roots by wet sieving (3). One hundred cm^3 of soil or 25 g of chopped roots were analyzed. Five instead of three stacked sieves were used (589 μm, 147 μm, 74 μm, 43 μm, and 43 μm apertures). Residues on the 74 μm and both 43 μm sieves were combined and diluted to 400 ml for a soil analysis and to 500 ml for a root analysis. From each dilution, 2-ml samples were examined for nematodes. The number of nematodes counted from soil samples was multiplied by 200 and reported as nematodes per 100 cm^3 soil. The number of nematodes counted from roots was multiplied by 1000 and reported as nematodes per 100 g roots. For comparison with the data from Laboratory A, the counts from roots were divided by 10, and the counts from soil were divided by two.

Nematodes. Three species of plant-parasitic nematodes (*Radopholus similis, Helicotylenchus multicinctus* and *Meloidogyne* spp. [J2]) were found in soil and roots at frequencies of occurrence large enough for comparison between the laboratories.

Statistical analysis. The consistency of the laboratory measurements was evaluated by two criteria involving, first, detection-nondetection of nematodes, and second, comparisons of data where the same nematode was counted in the matched samples by both laboratories. The laboratories analyzed stock samples that had been mixed and divided; thus the comparison of the data should demonstrate strong relationships at each step of the statistical analysis. The expected behavior of the data is given with each comparison.

First, the relationship in detection-nondetection of nematodes was evaluated with two-way frequency tables (BMDP program 4F) (4). The expected results from this tabulation would be near perfect agreement, i.e. both laboratories should detect the same species of nematode from the split samples, or both laboratories should not detect the species. The

majority of observations should occur in the frequency tables in the cells in the upper left (absent-absent) and in the lower right (present-present) (Fig. 1). The observations that are in disagreement are tallied in the cells on the other diagonal. The attained significance level of Yates' corrected chi-square should be small, providing strong evidence to reject the null hypothesis of independence between laboratories. Rejection would support the expected relationship between two laboratories. The percent agreement in detection-nondetection was calculated by adding the numbers in the diagonal cells of absent-absent and of present-present, dividing the sum by the sample size and then multiplying by 100. The expected percent agreemnt between laboratories should be high (>85%).

Second, the natural logarithms (ln) of the ratio of the counts from matched samples where both laboratories reported the presence of a species of nematode was calculated (BMDP program 2D) (4). The ln counts from laboratory A were subtracted from the ln counts from laboratory B, and a 95% confidence interval for the difference in the mean ln counts was calculated from a matched pair, t-test procedure. If the confidence interval for a species did not include zero and was shifted to the right of zero, we concluded that the counts from laboratory B were higher than the counts from laboratory A. If the confidence interval was

LAB B \ LAB A	ABSENT	PRESENT
ABSENT	ABSENT A ABSENT B	PRESENT A ABSENT B
PRESENT	ABSENT A PRESENT B	PRESENT A PRESENT B

Fig. 1. Frequency table for presence-absence data.

shifted to the left of zero, we concluded the reverse. The confidence interval yielded not only the matched pair t-test for the difference in the mean ln counts, but also gave an upper and lower limit for the amount by which one laboratory exceeded the other. The data should behave so that zero is included in each confidence interval and indicate that there is no evidence to support a difference between counts.

In our final analysis we plotted the ln of counts from matched samples where both laboratories detected the same nematode using bivariate scatter plots (BMDP program 6D) (4). This procedure provided a way to study the relationship of the counts from each laboratory. Even if the confidence intervals do not contain zero, the laboratories might still be consistently related, and this relationship could be employed to reconcile counts between laboratories. The data should provide high correlations of the ln counts ($r > 0.9$).

To justify this lower limit of 0.9 for r, we present a model for estimating the correlation between two independent measurements of the same quantity. Assume each of a total of n specimens of a species is randomly divided with one set of duplicates sent to each of two laboratories, A and B. Let A_i be the reading obtained on specimen i from laboratory A, and B_i the reading on the corresponding specimen from laboratory B with $i = 1, 2, \ldots n$. Assume:

$$A_i = T_i + \alpha_i$$
$$B_i = T_i + \beta_i$$

where: T_i = true reading on specimen i, with the variance of $T_i = v_t$; α_i = measurement error from laboratory A on specimen i having mean = 0 and variance = v_α; β_i = measurement error from laboratory B on specimen i having mean = 0 and variance v_β; (T_i, α_i, β_i) are mutually stochastically independent. Then the correlation, r, between A_i and B_i is expressed by

$$r = \frac{1}{\sqrt{1 + Q_A + Q_B + Q_A Q_B}}$$

where $Q_A = v_\alpha/v_t$, $Q_B = v_\beta/v_t$, reflecting the ratios of the measurement error variance to the variance of the 'true' readings.

If V_α and V_β approach zero, a close correlation ($r \approx 1.0$) would be obtained between the two laboratories. In fact, correlations exceeding 0.9 can be obtained even with an apparently high amount of measurement error. For example, if $Q_A = Q_B = 0.1$, then $r = 0.91$ and $\sqrt{v_\alpha/v_t} = \sqrt{v_\beta/v_t} = \sqrt{0.1} \approx 0.32$, which reflects the ratio of the error standard deviations to the standard deviations of the actual quantity. Thus, the error standard deviations can be as high as 32% of the true readings'

standard deviations, and still the correlation between the laboratories remains above 0.9. These computations remain the same when some additive biases are placed in the model:

$$A_i = T_i + a + \alpha_i$$
$$B_i = T_i + b + \beta_i$$

where a is the constant bias from laboratory A, and b is the constant bias from laboratory B. Bias may result from many sources, but remains constant for the laboratory. An example of bias would be consistent over or under counting of nematodes in samples.

The sample correlation cefficient, $\hat{r}$, is usd as an estimate of r and is computed from the observed pairs (A_i,B_i), $i = 1, \ldots n$.

$$\hat{r} = \frac{\sum_{i=1}^{i=n} A_iB_i - n(\bar{A})(\bar{B})}{\sqrt{\left(\sum_{i=1}^{i=n} A_i^2 - n(\bar{A})^2\right)\left(\sum_{i=1}^{i=n} B_i^2 - n(\bar{B})^2\right)}}$$

where $\bar{A}$ = the mean count of a species from laboratory A; $\bar{B}$ = the mean count of the same species from laboratory B.

RESULTS

Frequency tables. Both laboratories detected *R. similis* in all but one root sample. However, in the remaining analyses there was insufficient evidence to support consistency between the laboratories (Table 1). The detection of *Meloidogyne* spp. from soil was in extreme disagreement with a greater number of detections by Laboratory A. In general, the number of detections of nematodes from soil by Laboratory A was higher than that by Laboratory B. These results are indicated by the high tallies in the cell for present A-absent B (Table 1).

Detection-nondetection agreements. The agreement in detection-nondetection was at least 85% only for *R. similis* from roots (Table 1). For the remaining nematodes a confidence interval for agreement fell below 85%, and these measurements were considered inconsistent.

Ln ratios of counts. The counts from Laboratory B were higher than the counts from Laboratory A for *R. similis* and *H. multicinctus* as indicated by confidence intervals of the mean ln ratios (Table 2). The incidence of detections (present A-present B) for *Meloidogyne* spp. was insufficient to enable one to draw conclusions.

Plots of ln counts. The plots of the ln counts revealed no relationships between the laboratories to support consistency (soil with *R. similis*, $\hat{r}$ =

Table 1. Two-way frequency tables for detection (pres) and nondetection (abs) of nematodes from laboratories A and B.

Soil			Root		
Radopholus similis			*Radopholus similis*		
	abs A	pres A		abs A	pres A
abs B	0	121	abs B	0	0
pres B	2	77	pres B	1	199
	P = 0.3[y]			P = not determined	
	39 ± 7[z]			99.5 ± 0.01	
Helicotylenchus multicinctus			*Helicotylenchus multicinctus*		
	abs A	pres A		abs A	pres A
abs B	26	136	abs B	31	36
pres B	4	34	pres B	28	105
	P = 0.5			P < 0.01	
	33 ± 7			68 ± 7	
Meloidogyne spp. (J2)			*Meloidogyne* spp. (J2)		
	abs A	pres A		abs A	pres A
abs B	76	109	abs B	114	35
pres B	11	4	pres B	42	9
	P = 0.03			P = 0.5	
	40 ± 7			62 ± 7	

[y]The attained siqnificance level of Yates' corrected chi-square.
[z]Percent agreement in detection-nondetection of nematodes and 95% confidence interval. Percent agreement obtained by adding the diagonal cells abs-abs and pres-pres from the frequency table, dividing the sum by n and then multiplying by 100.

–0.02 ± 0.23* and *H. multicinctus,* $\hat{r}$ = 0.23 ± 0.34*, root with *R. similis,* $\hat{r}$ = 0.25 ± 0.14* and *H. multicinctus,* $\hat{r}$ = 0.35 ± 0.17* where * indicates approximate 95% confidence interval for r) (Fig. 2). *Meloidogyne* spp. was not considered because the number of concurrent detections was too small.

To illustrate the manner of serious treatment advocated for each component of a diagnostic protocol, we have adopted a possible model for selecting an appropriate dilution maxim that takes into account several criteria. The model may explain in part the discrepancy in nematode counts between the laboratories. Assume some number of nematodes, N, is randomly distributed in a suspension of volume D from which a sample of volume s is withdrawn for the purpose of counting the nematodes present. Let Q be the number of nematodes in this sample. Then a reasonable probability distribution for Q is Poisson with the property that mean and variance equal to (Ns/D). Whereas, Q may never

Table 2. Agreement in detection of nematodes by laboratories A and B and the mean of the natural logarithm (ln) of the ratio of counts from Laboratory B to Laboratory A.

Nematode	Detection agreement[z] (%)		Confidence interval of the mean of ln (B/A)
Radopholus similis	soil	39	0.55 ± 0.25
	root	99.5	1.20 ± 0.20
Helicotylenchus multicinctus	soil	17	0.66 ± 0.41
	root	53	1.67 ± 0.25
Meloidogyne spp. (J2)	soil	2	not determined
	root	5	not determined

[z]Percent detection agreement obtained by dividing the number in the cell for pres-pres in the frequency table by n and then multiplying by 100. n = 200.

be observed, a random variable, Q^*, is experimentally observed, and $Q^* = Q + E$, where E is the error incurred in counting the nematodes in the sample. Assume the mean of E is zero, and the variance (v) of E is a function of the mean (m). We estimated the error in counting nematodes on a 1-ml counting slide and found that as a function of m, $v = Ke^{\lambda m}$ where K and λ are positive constants (Fig.3). Recall that an equation of a straight line is expressed as $y = b + sx$, where s is the slope and b is the intercept. By taking the ln of the equation for v, the result can be written as $\ln v = \ln k + \lambda m$, which is the equation of a straight line where λ is the slope and ln k is the intercept. When ln v is regressed onto m, the slope of the regression is an estimate of λ (0.0058) and the exponential of the intercept of the regression is an estimate of K (2.20). Because $\frac{D}{s} Q^*$ is an unbiased estimate of N, the proportion of error is

$$P = \frac{Q^* D/s - N}{m} = \frac{Q^*}{m} - 1$$

The mean of P is zero, and the variance of P can be developed as a function of m

$$f(m) = \frac{m + v(m)}{m^2} = \frac{1}{m} + \frac{v(m)}{m^2} = \frac{1}{m} + \frac{Ke^{\lambda m}}{m^2}$$

Note that if there was no error in counting nematodes, then $f(m) = 1/m$. The best strategy in reducing the proportion of error in estimating

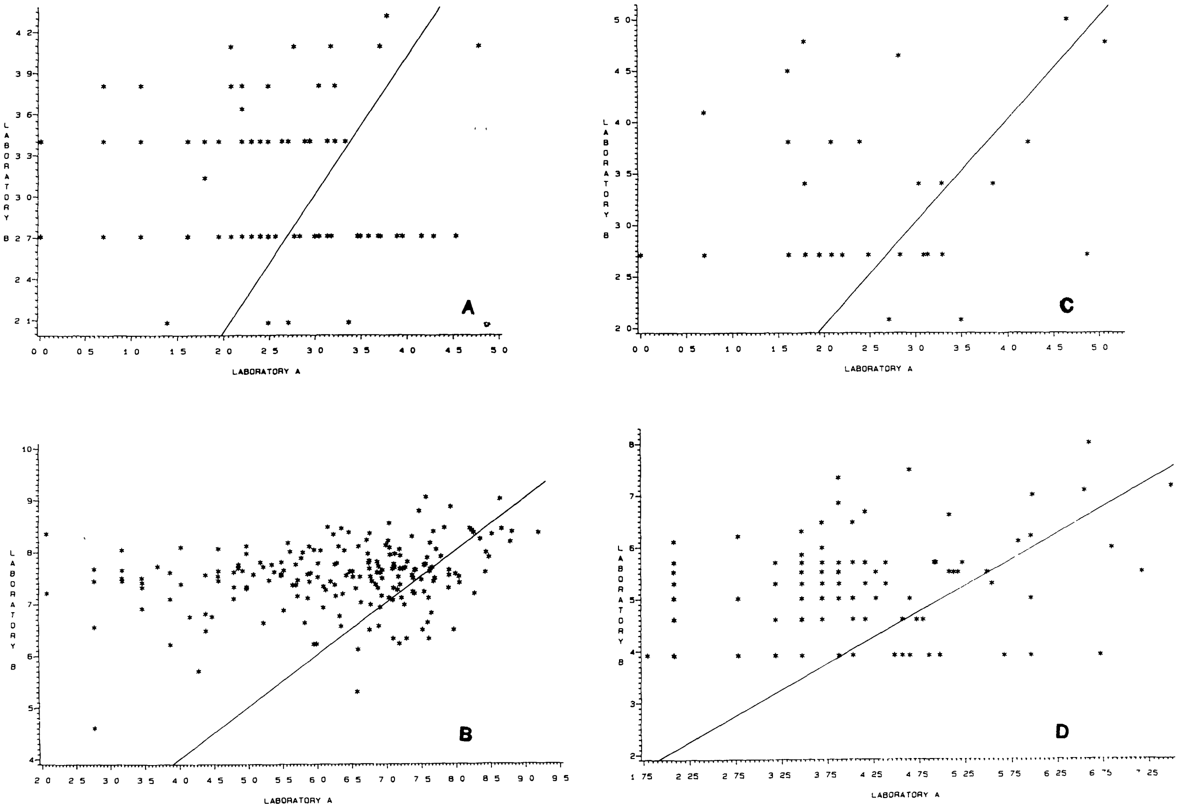

Fig. 2. Natural logarithms (ln) of the number of nematodes from laboratories A and B. A. *Radopholus similis* from soil. B. *R. similis* from roots. C. *Helicotylenchus multicinctus* from soil. D. *H. multinctus* from roots. The solid line is the line of identity where the ln of the number of nematodes from laboratory A equals that of laboratory B.

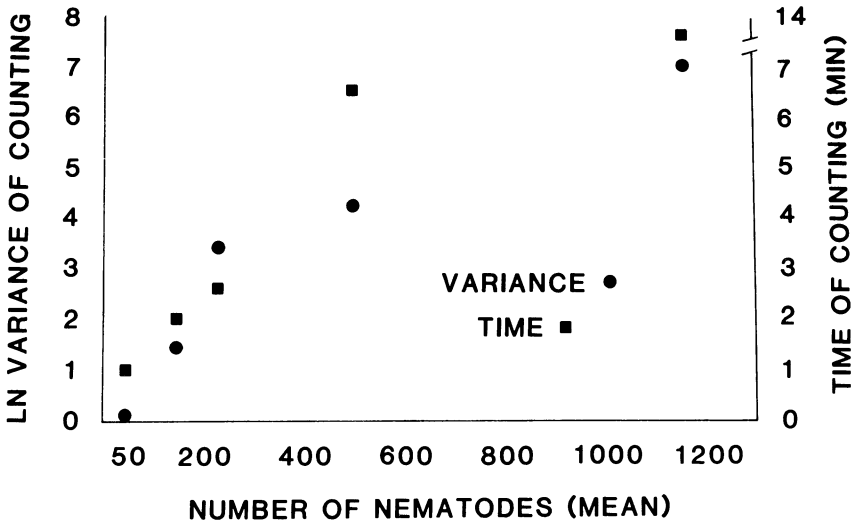

Fig. 3. Natural logarithms (ln) of the variance of counts of nematodes on a counting slide and the time of counting in minutes (n = 5).

N would be to obtain a sample having as many nematodes as possible, namely, to make m as large as possible. However, counting errors incrase as m increases (Fig. 3) so that the rapid rise of the counting error term conteracts the strategy of making m as large as possible; of course, when m is very large, the nematodes on a counting slide become too numerous to count.

The data in Fig. 3 were obtained by counting a single species of nematode from suspensions of 1200, 500, 225, 150 and 50 nematodes/ml that were placed on a 1-ml counting slide; the number of nematodes on each slide was counted five times. Time of counting presents an additional practical limitation (Fig. 3), but we have not included a time function in our calculations.

For a single sample the optimal value of m can be obtained using standard mathematical optimization methods. A closed form solution is unfortunately not possible in terms of K and λ. However the following iterative solution will converge rapidly to the value of m minimizing f(m)

$$m_{j=1} = \left(\frac{1}{\lambda}\right) \ln \left(\frac{m_j}{K(\lambda m_j - 2)}\right)$$

where j is the index of iteration. For K = 2.20 and λ = 0.0058, the value minimizing f(m) is m ≈ 843. this would be the optimum number of nematodes to be counted in a single sample estimate of any large N for the above value of K, with λ defining the measurement error.

Precision can be improved by taking more than one sample to estimate N. Let n be the number of samples of volume s to be obtained from the suspension of volume D. Then the average number of nematodes to be counted is the product of n and m, and the variance of the sample average proportion of error as a function of m given the number of samples (n) is

$$\text{variance} = f(m/n) = \frac{\dfrac{1}{m} + \dfrac{Ke^{\lambda m}}{m^2}}{n}$$

For a given variance $f(m/n) = f_e$, find the values of m and n minimizing the product of n and m. Standard mathematical optimization procedures yield the best value for m = average number of nematodes to count per sample as

$$m_o = \frac{1}{\lambda}$$

and the optimal number of samples as

$$n_o = \frac{1}{f_e}\left(\frac{1}{m_o} + \frac{Ke^{\lambda m_o}}{m_o^2}\right)$$

Now, for one sample the best value of m is 843 yielding a variance of (0.0016). If we set this variance = f_e, compute $m_o = 1/0.0058 \simeq 173$ nematodes per sample to be counted, then n_o is slightly less than 4. With the same precision, in other words, 692 from 4 samples (x173) is a smaller number to count than 843 from one sample. This model and our data yielding $\lambda = 0.0058$ suggest that samples actually used for counting should contain about 170 nematodes and that more than one sample be counted and averaged.

Dilution of a suspension of nematodes for counting can bring about substantial error at low nematode densities. The estimated error due to dilution and counting when using one sample at dilutions of 1 to 50 and 1 to 500 are plotted as functions of the sample size withdrawn for counting (s), the volume of the diluted suspension (D), and the number of nematodes in the suspension (N) (Fig. 4 and 5). The standard errors of the ratio of the observed count of nematodes (Q*) to the mean number of nematodes (m) in the diluted suspension are calculated by:

$$S.E. = \sqrt{\frac{1}{m} + \frac{Ke^{\lambda m}}{m^2}}$$

where m = Ns/D, K = 2.20 and λ = 0.0058.

DISCUSSION

Our analysis indicated no consistency between the counts of nematodes from the two laboratories. The agreement in detection-nondetection of nematodes from each laboratory was 85% for one of three nematodes from roots and none of three from soil. The one agreement proved upon analysis of the plots of the ln counts to be unrelated to one another. The disagreement might have involved errors from a number of sources, for example in subsampling, handling, extraction, identification, counting or reporting. Unfortunately, the sources of error cannot be identified with the information on hand. The gross differences obtained were not anticipated so known blank or duplicate samples were not submitted to the laboratories as standards, and quality control procedures were not reported by the laboratories. The potential for considerable error from dilution was established by our estimate of errors of dilution and counting.

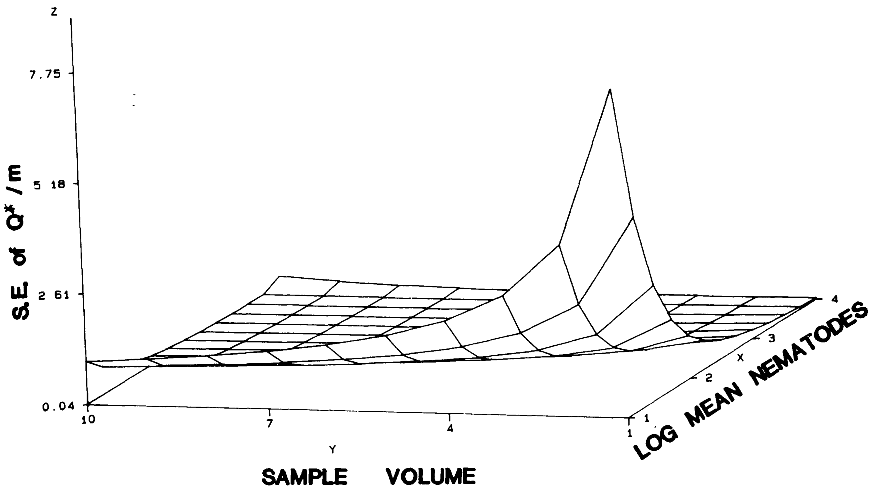

Fig. 4. Estimated error in counting nematodes when using one sample at a dilution of 1 to 50. Z is the standard error of the ratio of the observed count of nematodes (Q*) in the dilution suspension to the mean number of nematodes (m) in the dilution suspension. Y is the volume of the sample. X is the base 10 log of the mean number of nematodes in the dilution suspension.

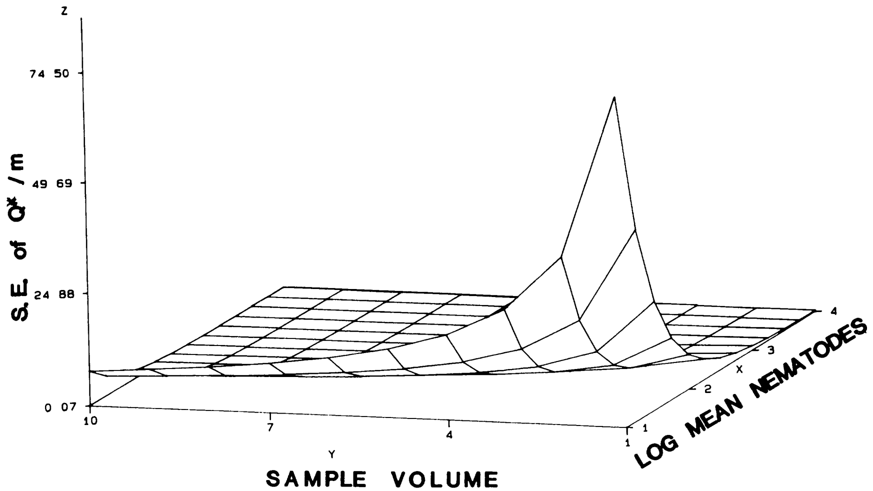

Fig. 5. Estimated error in counting nematodes when using one sample at dilution of 1 to 500. See Fig. 4 for descriptions of axes.

The errors of counting too few nematodes versus those of counting too many nematodes were addressed in a model that is intended as a first approximation to minimizing counting errors. This model suggests that differing dilutions should be used to obtain approximately the same number of nematodes per sample and that more than one sample be counted and averaged.

In the dilution procedures of both laboratories, only one sample was counted that contained the optimun number of nematodes that we estimated to minimize counting errors; in the remaining samples, the number of nematodes counted were well below optimum. The dilution procedure used by Laboratory B, counting a small sample from a large dilution, could have resulted in the large incidence of nondetection and detection with higher counts. Laboratory A also used a dilution procedure for the root analyses. The dilution procedures, therefore, could have accounted for part of the disagreement between the laboratories. Their contribution will need to be investigated with additional data that monitors all the operations of the extraction method and counting procedure.

Barker and Nusbaum (1) reviewed the difficulties and limitations facing diagnostics in nematology. Although several subsequent articles have defined the efficiency and reliability of extraction methods for specific cases (2,5,6), controlled laboratory extractions of different nematode species from different soil types demonstrated that five common extraction methods were exceedingly poor quantitatively (10). In practice even the cysts of *Heterodera schachtii* were not reliably extracted

from soil (9). Separation of nematodes from their substrate is critical because upon these determinations all other interpretations are dependent. There is urgent need in nematology to identify the inherent errors in all components of the diagnostic process leading to the final estimates of indigenous nematode populations. This framework will facilitate the establishment of a standard protocol for making reliable estimates of different nematode species and a quality control program to ensure that errors remain within acceptable limits.

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