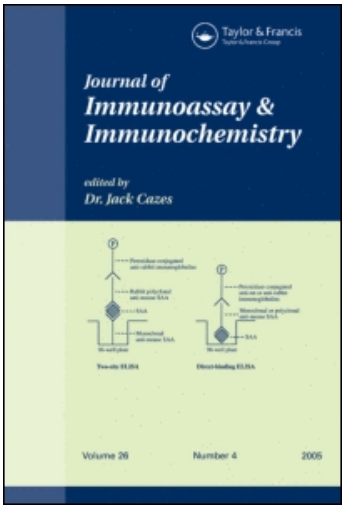

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COMPARISON OF SOME METHODS FOR DETERMINING CUTOFF VALUES FOR SEROLOGICAL ASSAYS: A RETROSPECTIVE STUDY USING THE FLUORESCENCE POLARIZATION ASSAY

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ABSTRACT

Different methods for determining cutoff values between positive and negative results for serological assays have been developed over time. Comparisons of some these methods show that five (Receiver Operating Characteristics, Frequency Distribution, and the mean, median and mode of the 100th percentile of a disease-free group of data) resulted in similar sensitivity (99.11%) and specificity values (99.21% to 99.58%). However, the Receiver Operating Characteristic analysis was considered more suitable, due to the ease of data manipulation and computer analysis, providing flexibility for different field applications.

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INTRODUCTION

Many studies have been published on the evaluation of sensitivity and specificity in developed, or in-use diagnostic tests. These attributes are easily influenced by the choice of the cutoff value which is the determined value used to separate positive from negative results for any given test or procedure(1). If the cutoff value is low, the sensitivity (the ability of the test to produce a positive result when the animal is diseased) will be higher(2) than the specificity (the ability of the test to produce a negative result from a known diseased-free animal).

Various methods have been developed to determine cutoff values; however, a comparison of these methods produced widely different results using data obtained with a serological test, — the fluorescence polarization assay (FPA), as an example. The evaluation of the FPA using the different methods resulted in different sensitivity and specificity values that impact on program cost and test acceptance.

This study compares some of the different methods (except kinetic methods for which data was not available) for determining cutoff values and their influence on sensitivity and specificity. Briefly, some of these methods are described below and presented in Table 1.

Statistical methods use a statistical parameter such as the mean, median, or the mode. As well, these methods may or may not incorporate the use of plus or minus one to three standard deviations to set the cutoff value. The mean, median, or mode could be chosen from the disease-free group or from the diseased group.

Arbitrary methods use a subjective cutoff value arbitrarily chosen, such as the mean plus or minus a number, which could be a percentage, an absolute value, or it could be a doubling of the mean of the disease-free group. The former method could be chosen using the disease-free group or the diseased group.

Receiver Operating Characteristic (ROC) curve plots all possible sensitivity/specificity pairs and graphically illustrates the effect of changing the cutoff value on the sensitivity/specificity pairs(3). The ROC curve generated allows for a comparison of two or more diagnostic tests without the problems of arbitrariness inherent in the calculation in single sensitivity/specificity estimates of other methods.(4)

Frequency distribution is another method that graphically organizes large masses of raw data into meaningful classes or categories(5) for both discrete and continuous data. This method determines the number of individual samples that belong to each class for the disease-free group and the diseased group. Using a tabular or graphical arrangement of the data by classes, the cutoff value can be visually estimated with reasonable accuracy.

Table 1. Different Methods for the Determination of a Cutoff Value for the FPA with Variations on Those Without References

Method	Cutoff Method	References
1	Mean of disease-free group + 1SD[1]	
2	Mean of disease-free group + 2SD	17
3	Mean of disease-free group + 3SD	18
4	Mean of diseased group - 1SD	
5	Mean of diseased group - 2SD	
6	Mean of diseased group -3SD	
7	Receiver Operating Characteristics	8,19
8	Double the mean of the disease-free group	18
9	Frequency Distribution	6
10	Mean of the disease-free group + 10	20
11	Mean of the disease-free group + 20	
12	Mean of the diseased group - 10	
13	Mean of the diseased group - 20	
14	P/N Ratio of diseased and disease-free groups	21
15	Comparison to the mean of the negative control	22
16	Mean[2] of 100th percentile of disease-free group	23
17	Median[3] of 100th percentile of disease-free group	18
18	Mode[4] of 100th percentile of disease-free group	

[1] Standard deviation (SD) is a measure variation about the mean.
[2] Mean is an average value which representative of a set of data. The value lies centrally within a set of data.
[3] Median is the middle value of a set of data arranged in order of magnitude.
[4] Mode is the value which occurs with the greatest frequency within a set of numbers.

Positive/negative (P/N) ratio, where P is the mean value of the FPA strong positive control divided by the sample FPA value. The individual P/N ratio for each sample was calculated using this method. The initial cutoff was determined using the mean of the strong positive control divided by the mean of the negative control.

EXPERIMENTAL

Serological Tests

The FPA was done as described by Nielsen et al.,(6) using *B. abortus* O-polysaccharide conjugated with fluorescein isothiocyanate (FITC) as the

antigen. The assay involved testing serum at 1:100 dilution in 2 mL of 0.01 M sodium phosphate, pH 7.4, containing 0.15 M NaCl, 0.1% sodium azide, and 0.05% lithium dodecyl sulfate (PBSAL), and measured in a fluorescence polarization analyzer (FPM-1, Jolley Consulting and Research Inc., Grayslake, IL, USA) to obtain a baseline measurement. Next, a predetermined amount of FITC-conjugated antigen in 0.01 M sodium phosphate, pH 7.4 containing 0.15 M NaCl and 0.1% sodium azide was added, mixed and incubated for approximately two minutes to allow for the interaction between the antigen and antibody. After incubation, the sample was again measured in a fluorescence polarization analyzer. In the presence of antibody, a high millipolarization (mP) result was achieved while, in the absence of an anti-Brucella antibody, a low mP value was obtained.

Test Sera

A total of 9224 sera were tested in the FPA. Of these, 8663 were obtained from Canadian cattle that were epidemiologically and serologically free of Brucellosis (the disease-free group) and 561 were obtained from cattle from which *B. abortus* had been isolated from milk or tissues or both (the diseased group).

Due to the sample size limitations of the Nonparametric Receiver Operating Characteristic (NPROC) software, a smaller subset of the disease-free sera, randomLy chosen, (n = 4437) was used to compare the two Receiver Operating Characteristic (ROC) programs (Table 3). The number of samples for the diseased group (n = 561) remained the same.

Data and Statistical Analysis

Data were analysed using different methods to set the cutoff value as presented in Tables 1 and 2. Some of these methods were variations of the same principle, such as the mean plus or minus 1, 2, or 3 standard deviations of either the disease-free group or the diseased group, respectively; the means plus or minus an arbitrary number (disease-free group or diseased group); the median, mean, or mode of the 100% percentile disease-free group, or the mean of data distribution disease-free group.

Receiver operating characteristics for parametric and non-parametric data were calculated using MedCalc Version 4.20(7) and Nonparametric Receiver Operating Characteristic Analysis Version 2.6.(8)

The chi-square test was used to determine goodness of fit of the data (P-value) used for the comparison of the two ROC software programs to a

Table 2. A Comparison of Some of the Different Methods for the Determination of a Cutoff Value for the FPA Sorted by Specificity of the Disease-Free Group in Ascending Order

Cutoff Method	Cutoff	Sensitivity	Specificity	Sens. + Spec.
Comparison to mean of negative control	75.10	100	27.23	127.23
P/N Ratio of both groups	3.316	100	47.85	147.85
Mean of disease-free group + 1SD[1]	80.0	99.64	90.36	190.00
Mean of disease-free group + 2SD	83.6	99.46	97.16	196.62
Mean of disease-free group + 10	86.5	99.29	98.68	197.97
Mean of disease-free group + 3SD	87.1	99.29	98.86	198.15
Mode[2] of 100th percentile of disease-free group	88.80	99.11	99.21	198.32
Median[3] of 100th percentile of disease-free group	89.80	99.11	99.49	198.60
Receiver Operating Characteristics	90.00	99.11	99.53	198.64
Mean[4] of 100th percentile of disease-free group	90.86	99.11	99.58	198.69
Frequency Distribution	90.86	99.11	99.58	198.69
Mean of disease-free group + 20	96.5	98.57	99.93	198.50
Mean of diseased group - 10	243.9	75.93	100	175.93
Mean of diseased group - 20	233.9	80.57	100	180.57
Mean of diseased group - 1SD	210.0	87.88	100	187.88
Mean of diseased group - 2SD	166.1	93.76	100	193.76
Double the Mean of the disease-free group	153.1	94.47	100	194.47
Mean of diseased group -3SD	122.2	96.97	100	196.97

[1] Standard deviation (SD) is a measure variation about the mean.
[2] Mode is the value which occurs with the greatest frequency within a set of numbers.
[3] Median is the middle value of a set of data arranged in order of magnitude.
[4] Mean is an average value which representative of a set of data. The value lies centrally within a set of data.

Normal distribution and calculation of the contingency coefficient (Yates correction for continuity was used). The contingency coefficient is a measure of association or dependence of classifications in a chi-square contingency table.

Cumulative frequency distributions were used to estimate deviation from the Normal distribution visually (Figure 2) for the data used in the comparison of two ROC software programs.

The sum of the sensitivity and specificity values were calculated (Table 2) to provide a numerical index of comparison.

RESULTS

The results presented in Table 2 show that comparison to the mean of a negative control (method 14) and the positive/negative ratio of the diseased group to the diseased-free group (method 15) produced a sensitivity of 100% with specificity values of 27.23% and 47.85%, respectively. The sensitivity decreased 0.36% from 100% for the mean of the diseased-free group plus 1 standard deviation (method 1) but improved the specificity to 90.36%. Minor variations in sensitivity occurred for the mean of the diseased-free group plus 2 or 3 standard deviations (methods 2 and 3) and the mean of the diseased-free group plus an arbitrary number of 10 (method 10) but increases to specificity were significant (6.8%-8.3%) in comparison to method 1.

Five statistically based methods: receiver operating characteristics, frequency distribution (Figure 1), mean, median, and mode of the 100th percentile of the diseased-free group all gave sensitivity values of 99.11% (methods 7, 9, 16, 17, and 18). These did not vary greatly in specificity, which ranged from 99.21% to 99.58%. In addition, these methods produced some of the highest summations of sensitivity and specificity with a difference of 0.37% between the lowest (198.32) and highest (198.69) sums.

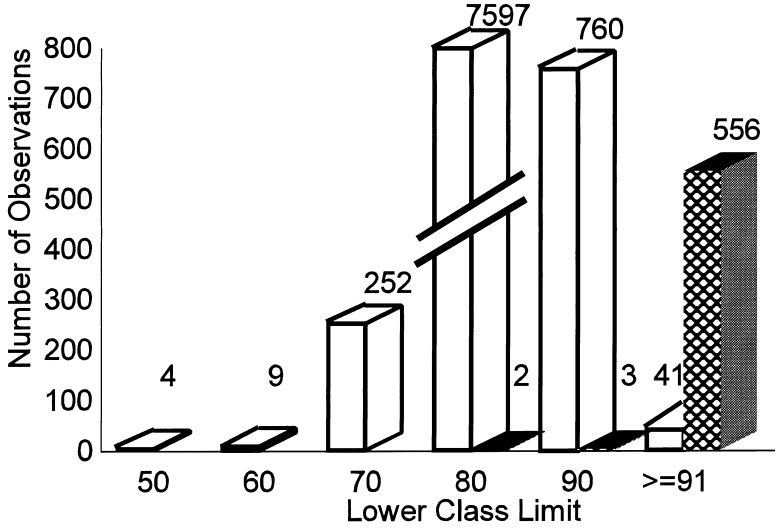

Figure 1. Frequency distribution of FPA data for the diseased group and the disease-free group. The open bars represent the disease-free group and shaded bars represent the diseased group.

Table 3. Comparison of Parametric and Non-parametric Software for ROC Analysis

Program	Cutoff	Sensitivity	Specificity	AUC[1]
Medcalc	91	99.1 (n = 561)	99.9 (n = 4437)	0.999
Non-parametric ROC	91	99.1 (n = 561)	99.7 (n = 4437)	0.999

[1] AUC = area under the curve.

Following the five statistically based methods were the means of the disease-free group plus 20 (sum = 198.50) and the diseased group minus 10 and 20 which depended on the correct arbitrary number being chosen (methods 11, 12, and 13). A slight improvement on the specificity. from 99.93% to 100%, resulted but the sensitivity values ranged from 75.93% to 98.57%. The sum of the disease-free group was 198.50 due to the higher specificity which compensated for the lower sensitivity.

The last four methods: mean of the diseased group minus 1, 2, or 3 standard deviations and double the mean of the diseased-free group (methods 4, 5, 6, and 8) gave specificity values of 100% but did not improve on the sensitivity values which ranged from 87.88% to 96.97%.

Data presented in Table 3 compares the sensitivity and specificity values calculated by 2 software programs for ROC analysis using a smaller subset of the same data, due to sample size limitations of the nonparametric ROC software. A chi-square test to test the goodness of fit of the data with a Normal distribution expressed a P value less than 0.05 suggesting that the distribution of the data may not approximate a Normal distribution and contingency coefficient (C) of 0.691 suggesting lack of association. Cumulative distribution plots are presented in Figure 2. Figure 2b suggests that the deviation from the Normal distribution was mostly with the diseased group.

The relative positions of the mean, median and mode of generic frequency distributions which are skewed to the right and left, respectively, are illustrated in Figure 3. For symmetrical distributions, the mean, median, and mode coincide.(5). The mean, median, and mode for the disease-free group (n = 4437) are 76.37 mP, 76.60 mP and 76.10 mP. The mean, median and mode for the diseased group (n = 561) are 253.9 mP, 267.0 mP and 268.4 mP. This indicates that both populations are slightly non normal, the diseased group being less non normal than the disease-free group. Since the slightly non normal data did not depart markedly from the normal as described by Hanley,(9) both programs gave similar results. Both software programs gave the same area under the curve (0.999), meaning that a

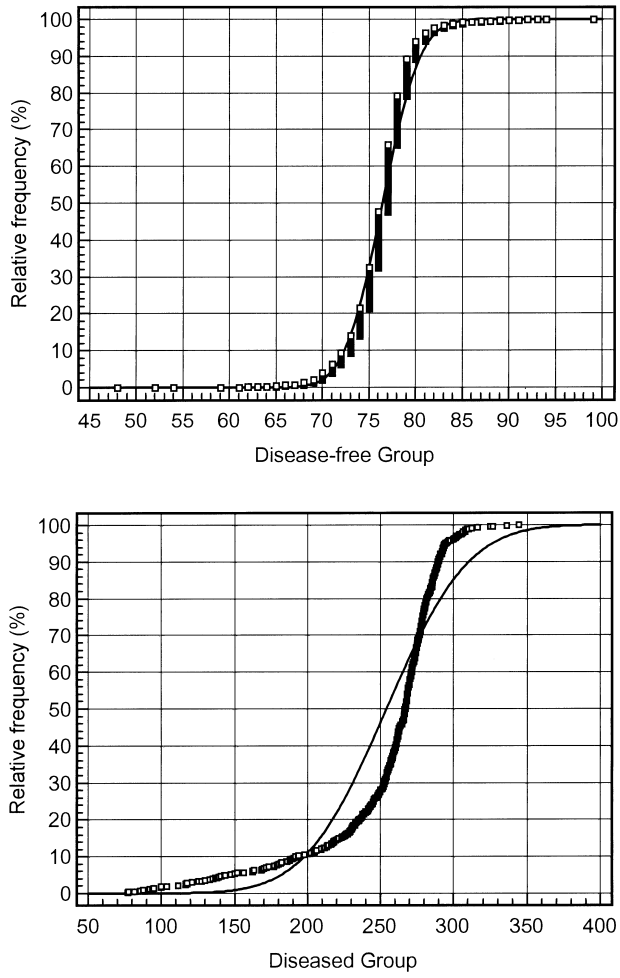

Figure 2. Cumulative distribution plots of the disease-free group and the disease group. The solid represents Normal distribution. The boxes represent the data distribution. Figure 2a represent the disease-free group (n = 4437) used for the comparison of the ROC programs. Figure 2b represents the diseased group (n = 561) used for the comparison of the ROC programs.

randomly selected sample from the diseased group has a test value greater than that of a randomLy chosen sample from the diseased-free group 99.9% of the time,(10) the same sensitivity (99.1%), but slightly different specificity values of 99.9% and 99.7%, respectively.

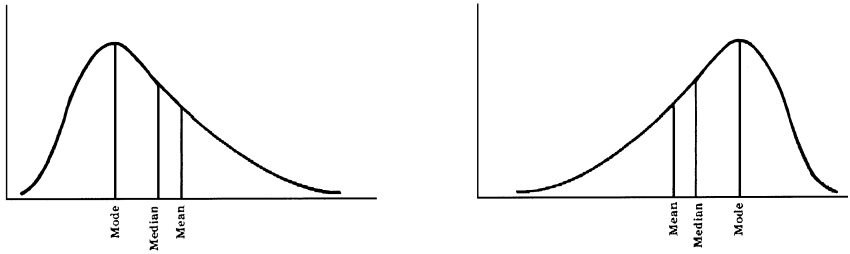

Figure 3. Illustrates generic asymmetrical distributions that are skewed to the right and left respectively.

DISCUSSION

Test performance can be described by four attributes: accuracy, precision, sensitivity, and specificity. Sensitivity is the ability of the test to produce a positive result when the animal is diseased. Specificity is the ability of the test to produce a negative result when the animal is not diseased. Accuracy is the ability of the test to identify positive and negative samples correctly, and is best described as sensitivity and specificity. Precision, or repeatability, is the ability of the test to produce consistent results in repeated tests. The former three attributes are influenced by the choice of cutoff value. An incorrect cutoff will have implications on program cost (repeat testing and labour), test acceptance (poor sensitivity and specificity), multinational harmonization of cutoffs, and trade.

A second consideration of test performance is the procedure of standardization and validation of a developed test. The variability of sensitivity and specificity from one population to another could be affected if the method for cutoff determination is not part of the standardization process. If the test is validated in several laboratories and the laboratories independently choose different methods to decide the cutoff, the sensitivity and specificity may be different, as presented in Table 2. This affects test acceptance, test harmonization, and trade. Thus, it is important for organizations involved in the development, standardization, and transfer of tests to standardize on a method to decide the cutoff.(11)

Several different methods for determining the cutoff values between positive and negative results for serological tests have been developed. Selecting the proper cutoff correctly is important, as this affects the accuracy (sensitivity and specificity) of the test in question. If the cutoff is set high, the sensitivity of the test decreases as in the last 6 methods presented in Table 2 (methods 4, 5, 6, 8, 12, and 13). The inverse is true of the specificity.(12) If

the cutoff is set too low, the specificity decreases as in the first 3 methods presented in Table 2 (1, 14, and 15)

Comparison of the test sample result to the negative control (method 15) is commonly used to determine positivity. Unfortunately, the success of this method is dependent on the correct choice of the negative control.

Using a pool of sera can mitigate this, rather than a single serum sample or the average of a number of sera. If a correct control can be chosen, a sufficient supply must be available for transfer of the test to other laboratories.

The coefficient of contingency (C) is a measure of relationship, association, or dependence of classifications in a chi-square contingency table.(5,7) The closer the C value is to 1 the greater is the degree of association. The C value for this study was 0.691 suggesting some association between the classifications for chi-square. The ratio method(14) can be more precise only if change in the diseased group is accompanied by a proportionate change in the disease-free group. This cannot occur unless a high degree of association exists. The success of this method is dependent on the relative change being the same for all biological samples, which is unlikely as shown by the lack of association.

Another approach has been the development of cutoffs based on the mean ± 1, 2 or 3 standard deviations for normally distributed data.(13) Data presented in Table 2 (methods 1, 2, 3) used this approach and improved on the specificity (90.36%, 97.16% and 98.86%) with less than 1% decrease overall in sensitivity from the 100% achieved by methods 14 and 15. However, incorrectly applying the above method without understanding the distribution of the data could lead to incorrect cutoff values. In this study, both populations are slightly non-normal, as previously mentioned. The mean, median, and mode for the disease-free group ($n = 8663$) were 76.56 mP, 76.70 mP, and 77.0 mP, respectively. This suggests that the mean could be used to decide the cutoff value; however, if the data had been more non-normal then the median or mode would be more applicable(7) in determining the cutoff value.

The percentile approach using the mode, median or mean of the 100th percentile (methods 16, 17, and 18) provided better specificity (99.21%, 99.49% and 99.58%, respectively) and sums of 198.32, 198.60, and 198.69 with a maximum difference of 0.37 between the highest and the lowest sum. However, this approach has disadvantages. The mean, median or mode can be affected by lack of data in the 100th percentile, for example, $n = 10$ compared with $n = 100$. As well, if the data is insufficient, one or two abnormally different results will affect the mean, median, or mode.

Frequency distributions (Figure 1) are more useful for the visual comparison of test accuracy, allowing for incremental shifts of the cutoff to

obtain an estimate of accuracy (minimal false negative and false positive results) regardless of the initial method chosen to determine the cutoff. The initial cutoff chosen for the frequency distribution in this study was determined using the percentile approach, so it is hardly surprising that the estimates of sensitivity and specificity are the same as the mean of the 100th percentile. Frequency distribution of the data is advantageous if it is used with other methods such as the mean of the 100th percentile of the diseased-free group but by itself as in Figure 1 is limited unless the data is contiguous and can be presented to 1 or more decimal places allowing for precise incremental shifts.

Using the mean plus or minus an arbitrary number is subjective providing no continuity for testing from one population of animals to another. Sensitivity for these methods was excellent (98.68% - 100%) but specificity decreased, accordingly, from 99.29% to 75.93% as in methods 10, 11, 12, and 13. Methods dependent on the correct choice of the arbitrary number are applicable to the original population used to evaluate the test but may not reflect the population to be tested,(3) for instance, in another area.

Doubling the mean of the diseased-free group (method 8) did not offer significant advantages over the other methods. The specificity of this method was 94.47%.

Unlike the above methods, ROC analysis determines all possible sensitivity/specificity pairs with associated cutoffs including the optimal sensitivity and specificity for both normal and non normal data that do not depart markedly from the normal(9) removing the arbitrariness and subjectivity inherent in the other methods. The merits of using parametric and nonparametric ROC approaches have not been clearly established for nonbinormal data. If the data is non-normal as it is in this study the diagnostic accuracy using the parametric approach could be distorted. A recent review the literature suggests that concern about bias or imprecision of estimates of the AUC should not be a major concern in choosing the parametric or nonparametric approaches(14) as the differences in the AUCs were very small for both approaches. A similar result was obtained in this study, in comparing ROC and NPROC. The AUCs in this study were both 0.999. Another study showed that parametric ROC analysis of laboratory data was acceptable even when the data may be decidedly non normal.(15)

The correct method chosen to determine the cutoff can affect test accuracy which, in turn, influences the program cost, acceptance, multinational harmonization, and trade. Receiver operating characteristics is more likely to improve the cost/benefit analysis of diagnostic decision making(16) than the other methods and removing subjective determination of the cutoff inherent in the other methods. For example, if the cutoff for the positive/negative ratio was chosen, this would result in a misdiagnosis of 52.15% of

the disease-free group compared with 0.47% for ROC analysis. Using ROC analysis can significantly reduce human and material resources in the cost of trace backs and repeat testing. Another benefit of ROC analysis is risk analysis. Since ROC analysis produces sensitivity/specificity pairs with associated cutoffs, it can be tailored to specific applications such as surveys, increasing sensitivity in the early stages of disease eradication, increasing specificity in the later stages of eradication, or disease monitoring programs once the disease is eradicated or under control.

The purpose of this study was to compare different methods for determination of a cutoff value and select the most suitable method. Receiver operating characteristics, frequency distribution, mean, median, and mode of the 100th percentile of the diseased-free group (methods 7, 9, 16, 17, and 18) provided the best overall sensitivity and specificity values. However, for ease of data manipulation and analysis, ROC analysis was the best method, providing the same sensitivity value (99.11%) as the other four methods and minimum difference in specificity (0.05% - 0.32%). Both ROC programs presented in Table 3 provide a range of cutoff values with associated sensitivity and specificity values, allowing for the optimal cutoff value for different field conditions.

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