Estimation of receiver-operating characteristic curves to determine accuracy of a competitive enzyme-linked immunosorbent assay for the serodiagnosis of Brucella infection in domestic water buffalo (Bubalus bubalis) and cattle

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Objective—To estimate receiver-operating characteristic (ROC) curves for a competitive ELISA (c-ELISA) that is used in serodiagnosis of brucellosis in water buffalo and cattle, to determine the most appropriate positive cutoff value for the c-ELISA in confirmation of infection, and to evaluate species differences in c-ELISA function.

Sample population—Sera from 4 herds of cattle (n = 391) and 4 herds of water buffalo (381).

Procedure—Serum samples were evaluated for Brucella-specific antibodies by use of a c-ELISA. On the basis of previous serologic test results, iterative simulation modeling was used to classify animals as positive or negative for Brucella infection without the use of a gold standard. Accuracy of c-ELISA for diagnosis of infection was compared between cattle and water buffalo by comparison of areas under ROC curves.

Results—A positive cutoff value of 30% inhibition for c-ELISA yielded sensitivity and specificity estimates, respectively, of 83.9 and 92.6% for cattle and 91.4 and 95.4% for water buffalo. A positive cutoff value of 35% inhibition yielded sensitivity and specificity estimates, respectively, of 83.9 and 96.2% for cattle and 88.0 and 97.4% for water buffalo. Areas under ROC curves were 0.94 and 0.98 for cattle and water buffalo, respectively.

Conclusions and Clinical Relevance—ROC curves can be estimated by use of iterative simulation methods to determine optimal cutoff values for diagnostic tests with quantitative outcomes. A cutoff value of 35% inhibition for the c-ELISA was found to be most appropriate for confirmation of Brucella infection in cattle and water buffalo. (Am J Vet Res 2003;64:57–64)
ple positive cutoff values and is often drawn nonparametrically, with a larger number of cutoffs improving quality of the resulting curve. These curves have been used in veterinary medicine to determine appropriate cutoff values for diagnosis of infection (or disease) in animals. In this manner, positive thresholds can be modified to fit particular testing schemes rather than relying on a single cutoff for all conditions. The area under a ROC curve is defined as the probability that a randomly selected infected animal will have a greater test result than a randomly selected noninfected animal, and is considered a measure of overall discriminating ability of the corresponding diagnostic test. To evaluate differences in ability of multiple tests to diagnose infection (or disease), areas under these ROC curves can then be compared.

Standard estimation of sensitivity and specificity, and, consequently, estimated ROC curves, depends upon knowledge of infection (or disease) status of animals tested. True status of animals is not always known, and therefore statistical procedures have been developed to estimate sensitivity and specificity of diagnostic tests without this information. Markov chain Monte Carlo (MCMC) simulation methods have been developed to solve complicated simulation problems and can be implemented for the evaluation of diagnostic tests.

The purpose of the study reported here was to estimate ROC curves for a c-ELISA that is used in the serodiagnosis of brucellosis in domestic water buffalo and cattle of Trinidad when true infection status is unknown. Diagnostic potential of the c-ELISA was compared between species by use of area under the ROC curves. A secondary objective was to determine appropriate cutoff values for confirmation of B. abortus infection in domestic water buffalo and cattle of Trinidad. Cutoff values were selected on the basis of a high specificity and Youden index (sensitivity + specificity – 1).

Materials and Methods

Sample selection—During 1999, 8 herds in Trinidad were selected for evaluation of brucellosis serologic tests. Four of these herds (2 herds of domestic water buffalo, 1 herd of Bos indicus cattle, and 1 herd a mixture of Bos taurus and Bos indicus cattle) were considered Brucella-infected on the basis of the results of the government screening program. Four additional herds (2 herds each of cattle and water buffalo) believed to be free of brucellosis based on epidemiologic factors (lack of reported abortions in historically closed herds) or previous testing were also selected for evaluation.

Separate herds in the statistical model are not allowed to have the same infection prevalence when estimating diagnostic test characteristics without the use of a gold standard (ie, knowledge of true infection status). Thus, data from the 2 herds of cattle that were believed to be free of brucellosis were combined to form a single population for the purpose of analysis. Data from the 2 herds of water buffalo that were believed to be free of brucellosis were also grouped together before analysis. Therefore, data were considered to be from 6 populations—3 cattle and 3 water buffalo.

Blood samples were obtained from animals on study farms from April through June 1999 as part of the brucellosis-screening program instituted by the Trinidad and Tobago government. A minimum of 100 animals that were ≥ 1 year of age, or the entire herd if there were < 100 animals, were tested from each farm. Whole blood samples were allowed to clot overnight at 4°C before centrifuging and harvesting of sera. All samples were collected, and aliquots were stored at −40°C until serologically tested.

Serologic test results in these animals concerning 4 traditional agglutination tests (standard plate agglutination, card, buffered plate agglutination, and standard tube agglutination tests) have been reported. The c-ELISA was performed on banked sera in September 2001 after necessary equipment and reagents were acquired.

c-ELISA for the diagnosis of Brucella infection—The banked serum samples previously tested by the 4 agglutination procedures were subsequently evaluated for Brucella-specific antibodies by use of a c-ELISA. The established protocol by Nielsen et al with minor modifications was used for all testing. In brief, 100 µL of Brucella smooth-lipopolysaccharide antigen diluted (1 µg/mL) in carbonate buffer (pH, 9.6) was added to all wells of 96-well flat-bottom microtiter plates. Plates were incubated at 25°C for 18 hours to bind antigen and then stored at −40°C until use. Antigen-coated plates were allowed to thaw at room temperature (approx 25°C) and washed 4 times with PBS solution (pH, 7.2) that had 0.05% Tween 20 added. Test and control sera were diluted 1:10 with 50:50 (vol/vol) 0.015M EDTA/0.015M ethylene glycol-bis[β-aminoethyl ether]-N,N,N',N'-tetraacetic acid buffer (pH, 6.3). Control samples (ie, positive, negative, sera from vaccinated animals, and buffer solution only) were added to specified locations on the microtiter plate in a volume of 50 µL. Fifty microliters of diluted test sera was then added to remaining wells of the plate in duplicate. To each well, 50 µL of a 1:6,000 dilution of murine anti-smooth-lipopolysaccharide monoclonal antibody with O-polysaccharide side chain specificity was immediately added following test and control sera. The test plate was placed on a microtiter plate shaker for 5 minutes and then sealed and incubated for 30 minutes at 25°C. Following this first incubation, the plate was washed 4 times with PBS solution (pH, 7.2) with 0.05% Tween 20. To each well of the microtiter plate, 100 µL of a conjugate solution containing 1:3,000 dilution of goat antimouse IgG antibody conjugated with horse-radish peroxidase was added. The plate was sealed and incubated for 30 minutes at 25°C. The test plate was washed 4 times with PBS solution (pH, 7.2) with 0.05% Tween 20 after completion of this incubation. Substrate solution containing 3% hydrogen peroxide and 0.040M 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) was added to all wells of the plate, and the plate was sealed and incubated for 30 minutes at 25°C. Following this incubation, the plate was washed 4 times with PBS solution (pH, 7.2) with 0.05% Tween 20. To each well of the microtiter plate, 100 µL of a conjugate solution containing 1:3,000 dilution of goat antimouse IgG antibody conjugated with horse-radish peroxidase was added. The plate was sealed and incubated for 30 minutes at 25°C. The test plate was washed 4 times with PBS solution (pH, 7.2) with 0.05% Tween 20 after completion of this incubation. Substrate solution containing 3% hydrogen peroxide and 0.040M 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) was added to all wells of the plate and allowed to react for 40 minutes by use of a microtiter plate shaker. Absorbance at 410 nm was measured by use of an ELISA plate reader at completion of the 10-minute incubation-mixing period. Test results were accepted as valid if control sera absorbance on each plate fell within ranges specified in the published protocol.

Proportion inhibition (I) was calculated as follows:

\[
I = \frac{\text{mean OD of conjugate control sera} - \text{mean OD of test sera}}{\text{mean OD of conjugate control sera}}
\]
The Bayesian evaluation of test parameters without a gold standard allowed for the estimation of predictive probabilities that were then used for simulation of infection status. Comparison of the sensitivity and specificity of the 4 agglutination tests generated predictive value positive (PVP) information for all possible agglutination test result patterns (probability of infection given test result pattern). Simulation methods based on these results were then used to assign infection status to each animal under study by use of MCMC procedures. Resulting MCMC iterates (values for each parameter of interest) are random but dependent upon previous selections, thus resulting in a chain of values. This distinguishes this method from similar Monte Carlo procedures that produces independent selections.

Implementation of these MCMC methods necessitates estimation of probabilities of infection that should be derived from information independent of the diagnostic test under evaluation (ie, c-ELISA). The probability of an animal having a specified agglutination test result pattern (eg, positive results for all 4 tests) is equal to the probability of having an infected animal with that pattern plus the probability of having a noninfected animal with that same test pattern. When an animal has a particular test result pattern, a generalized PVP function can be defined as the conditional probability that an animal has the infection and is calculated as the proportion of the total probability contributed by infected animals. Calculation of a general PVP function is dependent on test sensitivity (Se), specificity (Sp), and infection prevalence (Pr) and can be written for a simplified situation involving results of 2 tests where both results were positive as follows:

\[
PVP = \frac{Se_1 \times Se_2 \times Pr}{[Se_1 \times Se_2 \times Pr] + [(1 - Sp_1) \times (1 - Sp_2) \times (1 - Pr)]}
\]

The numerator is composed of the probability that the animal was truly infected, and the denominator is the sum of the probabilities that the animal was infected and noninfected. This simplified equation also assumes conditional independence of test results. The PVP function was modeled for the agglutination test results of all animals under study and used for the simulation of infection status (Table 1).

The original 391 c-ELISA values from the sera of cattle were resampled by making 391 selections from the original values randomly with replacement. This means that all original c-ELISA values had an equal probability of being chosen for each of the 391 new selections. This created a new collection of 391 c-ELISA values, forming what is often referred to as a pseudosample because it does not represent a true sample of the population but a resampling of a sample. The same procedure was performed for the 381 water buffalo c-ELISA values. Therefore, each pseudosample contained the same number of c-ELISA values as the original sample, and these were used for the subsequent estimation of sensitivity and specificity. This is similar to performing a bootstrap analysis, when such pseudosamples are repeatedly drawn to account for the variability in observations when the true underlying distribution of the observations is unknown. The bootstrap approach has been used to obtain confidence limits for sensitivity and area under the ROC curves for diagnostic tests with quantitative outcomes. Bootstrap sampling has also been used to evaluate diagnostic tests when only partial gold standard information was available. The bootstrap approach is not constrained by assumptions relating to the distributional form of the c-ELISA values (eg, binormal) that are necessary in other procedures used to estimate interval limits.

All animals included in these pseudosamples of c-ELISA values were subsequently classified as infection positive or negative based on Bernoulli sampling with success probability (infection positive) equal to the PVP function for their corresponding agglutination test result pattern. This simulated infection status was used as if it were the true (unknown) status for all calculations. Classification of animals in this manner provided the information necessary for estimation of sensitivity and specificity.

Table 1—Predictive value positive (PVP) estimates determined on the basis of agglutination test results and Markov chain Monte Carlo simulations for 6 cattle and water buffalo populations in which the true Brucella infection status was unknown

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<th>CT</th>
<th>BPAT</th>
<th>STAT</th>
<th>PVP estimates* for cattle populations</th>
<th>PVP estimates* for water buffalo populations</th>
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*Point estimate for the predictive value positive function is the median of the posterior distribution rounded to 2 decimal places. Estimated infection prevalences were 0.08, 0.00, 0.14, 0.00, 0.56, and 0.34 for populations 1 through 6, respectively, on the basis of previously reported findings. SPAT = Standard plate agglutination test. CT = Card test. BPAT = Buffered plate agglutination test. STAT = Standard tube agglutination test. P = Positive results. N = Negative results.
specificity of the c-ELISA for the detection of Brucella infection at multiple proportion inhibition cutoff values (positive thresholds).

Sensitivity and specificity of the c-ELISA were determined for 99 possible cutoff values ranging from 0.01 to 0.99 proportion inhibition at 1% intervals. Sensitivity (probability of a positive test result in an infected animal) was calculated as the proportion of simulated infected animals with c-ELISA results greater than or equal to each cutoff value out of the total number of infected animals. Specificity (probability of a negative result in a noninfected animal) was calculated as the proportion of simulated noninfected animals with c-ELISA results less than each cutoff value out of the total number of noninfected animals.

Area under the estimated ROC curve was calculated by 2 simple geometric methods through use of the point estimates for sensitivity and specificity of the c-ELISA at all evaluated cutoffs. Areas of adjacent rectangles obtained by multiplying sensitivities (height) by difference in successive specificities (base width) for each cutoff were summed as a nonparametric estimate of area under the ROC curve. Summation of areas corresponding to adjacent trapezoids formed by the mean value of successive sensitivities (mean height of trapezoid) and difference in successive specificities (base) was also used as a nonparametric estimate of area under the ROC curve. These 2 numeric approximation methods are used in calculus to estimate area under curves when explicit integration is not possible.

Area under the ROC curve can be defined as the probability that a randomly selected infected animal will have a greater test result value than a similarly selected noninfected animal. This relationship was used for area estimation by use of the resampled c-ELISA values from cattle and buffalo (pseudosamples). Each c-ELISA value from all classified infected animals was compared with all values from noninfected animals. A value of 1.0 was assigned toward estimation of the area under the ROC curve if the infected animal had a greater c-ELISA value than the noninfected animal. A value of 0 was assigned if the result of the noninfected animal was greater than the results of the infected animal, and a value of 0.5 was assigned if c-ELISA results were equal. The area under the ROC curve was then estimated as the mean of all possible comparisons.

The MCMC technique was implemented by use of available software to assign infection status and iteratively estimate all parameters of interest. The aforementioned random selection of c-ELISA values (pseudosamples), estimation of PVP functions, and assignment of infection status for all animals were performed at each iteration of the MCMC procedure. On the basis of the simulated infection status at each iteration, sensitivity and specificity of the c-ELISA for the diagnosis of Brucella infection were calculated for the 99 selected cutoff values. The model was also used to estimate area under the generated ROC curve corresponding to each iteration.

Computer code was designed so that the ROC curves and corresponding areas under the curves for use of the c-ELISA in water buffalo and cattle were independently calculated. Species-specific evaluations were nested so that at each iteration of the MCMC procedure, values for all parameters in both species were produced. Areas under the ROC curves of cattle and water buffalo were also subtracted from each other, and distribution of these differences was used to evaluate the probability that test functioning was species-dependent. Nonparametric estimations of areas under the ROC curves were based on point estimates for sensitivity and specificity obtained from the entire sample of MCMC iterates and therefore were not performed at each iteration.

Plots of model parameter iterates (eg, sensitivity and specificity) were monitored for trends in successive iterations to determine when convergence was achieved. Convergence was considered the point when parameter values (iterates) no longer drifted over successive iterations and thus appeared to be fluctuating randomly without discernible pattern. Convergence was then confirmed by calculating the Gelman-Rubin statistic and the Geweke diagnostic through use of available software programs. Values obtained prior to reaching convergence, termed the burn-in phase of the analysis, were not used for making inferences. The first 20,000 iterations were discarded as the burn-in phase, and inferences for area under the ROC curves and sensitivity and specificity estimates were made on the basis of the subsequent 200,000 iterations. Median values and percentiles were used as point estimates and probability intervals, respectively, for the ROC curves of the c-ELISA. Diagnostic accuracy of the c-ELISA for the diagnosis of Brucella infection at each potential cutoff value was determined by calculating the Youden index (sensitivity + specificity – 1) for cattle and water buffalo.

Results

c-ELISA results—Seven hundred and seventy-two animals in Trinidad were serologically evaluated for B
abortus-specific antibodies by use of the c-ELISA as well as the 4 previously reported agglutination tests.° Serologic data were obtained for 391 cattle from 3 populations and 381 domestic water buffalo from 3 populations. For descriptive purposes, each animal was classified as infected or noninfected on the basis of agglutination test results if the median of the MCMC simulated PVP function was $> 0.5$ or $< 0.5$, respectively. The distribution of c-ELISA results for the 772 animals on the basis of this classification was created (Fig 1) and indicated that 92% (116/126) of infected animals had c-ELISA results $> 0.5$ or $< 0.5$; respectively, of 30% inhibition cutoff (95% interval) and 97.4% for water buffalo. Area under the ROC curve for the c-ELISA was estimated to be 0.94 and 0.98 for cattle and water buffalo, respectively, using median values of the posterior distributions obtained from MCMC simulation (Table 3). These estimates did not represent a probable difference between species on the basis of the corresponding 95% interval, which included 0. Simple geometric methods produced similar estimates of the area under the ROC curves (ie, 0.93 for cattle and 0.98 for water buffalo).

**Discussion**

Receiver-operating characteristic curves can be used to compare overall discriminating ability of diagnostic tests among groups of animals over the range of all possible cutoff values. A test with no discriminating ability would have an area under the curve of 0.50; that is, a randomly selected infected animal would have a greater test result than a similarly selected noninfected animal only 50% of the time. A perfect test would have an area of 1.0, meaning there was complete separation of test results for infected and noninfected animals. Estimated areas under the ROC curves for the c-ELISA had good to excellent discriminating ability, as areas for ROC curves of cattle and water buffalo were close to 1.0 (0.94 and 0.98, respectively).

Areas under ROC curves for cattle and water buffalo were not significantly different, suggesting equal diagnostic potential in both species. Previous work
evaluating serologic tests in water buffalo of Trinidad revealed differences in diagnostic sensitivity and specificity of brucellosis tests between cattle and water buffalo. Results of our study failed to demonstrate differential accuracy for the c-ELISA in the diagnosis of Brucella infection in cattle and water buffalo. This may be the result of differences in the tests evaluated. Previously evaluated screening tests were secondary binding assays where test sample antibody must first bind to antigen (whole Brucella cells) and then functionally form visibly apparent agglutination reactions. Ability of water buffalo antibody from infected animals to agglutinate B abortus may not be the same as antibody from infected cattle. The c-ELISA is a primary binding assay where test sample antibody competes for antigen-binding sites with monoclonal antibody added during the testing procedure. Water buffalo antibody from infected animals may be able to block binding of monoclonal antibodies with the same efficiency as cattle antibodies.

Evaluating areas under ROC curves is a common method to compare overall diagnostic accuracy of a test. This comparison can be flawed, however, when estimated curves intersect. Therefore, it is theoretically possible for a test to function quite differently in 2 species of animals even if the areas under the ROC curves are not significantly different. Estimated ROC curves in our study did cross, but this was not expected to unduly affect the comparison because the ROC curve of the water buffalo was consistently higher than that of cattle, except at the larger cutoff values.

The c-ELISA for the diagnosis of Brucella infection is a quantitative test. Results are reported as percent inhibition of test sample, compared with conjugate control without serum or competing antibodies. This allows for evaluation of multiple positive thresholds to adjust diagnostic potential for specific testing conditions. Nielsen et al recommended a positive cutoff value of 30%, which yielded estimates of sensitivity and specificity of 83.9 and 92.6%, respectively, for cattle in our study. Corresponding estimates obtained for water buffalo were 91.4 and 95.4% for sensitivity and specificity, respectively. Mean values of the Youden index peaked at a cutoff of 35%, and this cutoff yielded estimates of specificity of 96.2 and 97.4% in cattle and buffalo, respectively. High specificity is an important criterion for confirmatory tests. Diagnostic potential was not found to be different between species on the basis of area under ROC curves; therefore, a single cutoff value that maximized mean Youden index with high specificity was preferred. This led to selection of a positive threshold of 35% inhibition as most appropriate for the brucellosis control program of Trinidad.

Iterative simulation techniques can be used to estimate ROC curves for diagnostic tests in absence of a gold standard. These methods necessitate availability of other information such as results from alternate tests, but the methods are especially useful for situations when a gold standard test is too expensive or invasive to perform. Some diseases, such as brucellosis, require bacterial isolation to confirm infection, and this may not be effective at determining true status in all animals. Bacteriologic methods are not expected to be 100% effective at recovering viable organisms from infected animals. Animals in which isolation techniques are successful may be different in stage or degree of infection, compared with infected animals that have negative bacterial culture results. False-positive culture results occur very infrequently; however, false-negative results could have a meaningful effect on estimates of diagnostic test accuracy.

Simulation methods have the advantage of allowing each animal to have a probability of being infected rather than relying on imperfect information from a flawed gold standard test. Probability of infection must be determined by use of information obtained from another source, and in our study, we used information concerning results from 4 traditional agglutination tests (ie, standard plate agglutination, card, buffered plate agglutination, and standard tube agglutination tests). These methods provide a statistical solution to the problem of estimating diagnostic test parameters when true infection status is unknown. However, confirmation of these methods may require their implementation in samples of animals where knowledge of true infection status is available.

The model used in our study was a mixture of Bayesian and nonparametric techniques. Bayesian methods were used to iteratively assign infection status to all animals under study, and then the characteristics of the c-ELISA were evaluated nonparametrically. This model accounted for variability through the probabilistic assignment of infection status as well as the selection of random pseudosamples of c-ELISA values to use for estimation of sensitivity and specificity at each iteration. Appropriateness of the resulting probability intervals for the estimates is difficult to ascertain, as this blending of methods has not been previously reported in this field of study. Point estimates for diagnostic test parameters are not expected to be adversely affected (biased) by this combination of statistical methods, because similar point estimates were obtained when the analysis was performed without the selection of pseudosamples. Removal of this nonparametric component, however, resulted in conspicuously narrow intervals that did not account for variability in the c-ELISA values.

Medians of the posterior distributions were used as point estimates for evaluation of the c-ELISA in cattle and water buffalo. The sample means can also be used as point estimates, and the decision to use 1 instead of the other is often determined through visual observation of posterior distributions and investigator preference. The mean is well suited for unimodal, symmetric distributions. The median may be a better choice of a point estimator when the posterior is not perfectly symmetrical. The medians and means of the posterior distributions were similar for this analysis, and either could have been used as a valid point estimate. Sensitivity and specificity are probabilities, and as such their values are constrained to be between 0 and 1. When the mode of a posterior is near the maximum or minimum value, the distribution will necessarily be skewed to some degree. This was the case for many of the diagnostic test parameters; therefore, medians of the posterior distributions were reported.
Diagnostic test parameters such as sensitivity and specificity are important aspects of confirmatory tests used for controlling infection in populations, and knowledge of test accuracy at different cutoff values is imperative when designing eradication programs. Traditional approaches use a gold standard for determination of diagnostic test characteristics. However, a gold standard is often not available as a result of study limitations or inability to determine true infection status in an economically and ethically feasible manner. Simulation methods using iterative techniques can be used to plot ROC curves and estimate overall accuracy of diagnostic tests when information from a gold standard is not available. Additionally, these methods may be most appropriate for evaluation of tests with quantitative outcomes where traditional research methods are exceedingly difficult or impossible to implement.

References