VALIDATING A BOVINE BRUCELLOSIS ELISA TEST FOR APPLICATION IN URUGUAY

M. SILVA, G. MULLER, F. ERRICO, M. DILAVE
Rubino, Montevideo, Uruguay

Abstract

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Sera from 600 cattle on Rio Negro Island, known to be free of brucellosis, and 400 sera from vaccinated cattle but known to be negative in the Rose-Bengal test were selected for validation of the FAO/IAEA test kit for detection of antibody to Brucella abortus. Two conjugates, one a polyclonal antiserum and the other a monoclonal antibody, were evaluated. When evaluated for reproducibility using the sera from uninfected cattle, the average coefficient of variation for duplicate samples was 7.1% ± 5.5. The serum control samples did not exceed OD limits as established for the kit, for any of the 15 plates evaluated. When evaluated by regression analysis, the control sera had an average correlation coefficient of 0.996, indicating a high degree of agreement between the observed OD values of controls on each plate vs the expected values for those controls. Specificity in the assay was >98% as calculated by the PP or regression methods. Comparison of the monoclonal and polyclonal conjugates using sera from vaccinated cattle indicated that many of the cattle must have been vaccinated as adults because of high antibody levels detected by both conjugates. Before this assay can be used on vaccinated animals, the kit will have to be evaluated using sera from animals of known age of vaccination.

1. INTRODUCTION

As in many Latin American countries, brucellosis was a major problem in Uruguay [1, 2]. In 1965 animal health authorities established a National Control Plan based on vaccination, serodiagnosis, and slaughter of seropositive animals. Strain 19 vaccination (9 x 10^9 organisms per dose) was used in heifer calves and serodiagnosis was accomplished using the Rose-Bengal test (RBT) as the screening test followed by the 2-mercaptoethanol (2ME) and the Complement Fixation test (CFT) as a confirmatory tests. Currently, RBT and the rivanol confirmatory test are being used. Positive reactors on the screening and confirmatory tests continue to be systematically removed from the farms and slaughtered. Because of this intensive program during the past 30 years there have been neither abortions nor bacteriological isolation of Brucella abortus in the country.

Currently, the estimated prevalence of bovine brucellosis in dairy cattle is 0.2% and in beef cattle approximately 1% [3]. An efficient serodiagnostic assay applied to a disease of such low prevalence requires that the test be highly sensitive if the predictive value of a positive test result is to be of any value. Non-specific responses that give false-positive results must be avoided. However, antibody from vaccination in heifers may interfere with the test. This is particularly true when the vaccine is given to animals older than 9 months of age, which occurs in an estimated 10% of heifers [4]. Authorities in Uruguay are considering eliminating vaccination because of its interference in seroassays and therefore its detrimental effects in attempts to certify the country free of brucellosis. The objective of this work was, to confirm a highly specific and sensitive test that may be used either as a screening test or a confirmatory test for brucellosis. We evaluated the FAO/IAEA ELISA kit for use under Uruguayan epidemiological conditions.

2. MATERIALS AND METHODS

Sera from dairy cattle heifers of known Brucella abortus infection/vaccination status were selected for use in assessing an ELISA (FAO/IAEA test kit) for bovine brucellosis. Six hundred sera of non-infected cattle from the non-endemic Rio Negro island were selected at random and 400 sera from vaccinated cattle, negative on the Rose-Bengal test, were also evaluated. The age of vaccination of these cattle was unknown.

Two versions of the ELISA test kit were used. The first employed a polyclonal conjugate whereas the second utilized a monoclonal conjugate with specificity for bovine IgG1. The kits were used as described in the kit instructions with the exception that the monoclonal conjugate had to be retitrated to achieve the desired reactivity: it was used at 1:4000 rather than the recommended 1:11000 as specified
in the instructions. The polyclonal test kit was assessed for reproducibility between wells of the microlitre plate and between plates. Cut-offs were determined and compared for non-infected/non-vaccinated and vaccinated animals for the polyclonal version of the kit, and for only vaccinated animals on the monoclonal-based kit.

The data were analyzed following normalization by two methods. The ODs of each sample were converted to percent of the high positive control (PP) as described in the test kit. This method relies on a control against which tested samples on the plate are normalized. The data were also normalized by regression analysis, which uses a standard curve and the weight of all 4 control samples as the basis for normalizing all test sera. For regression analysis, the mean OD (n = 60) for each control serum, derived from all 15 plates, was determined and designated the "expected OD value" for that control. A standard curve for each plate was determined by plotting the ODs of the control samples for that plate against their expected OD values by linear regression analysis. All test sample ODs for that plate were then normalized by extrapolation from the standard curve. Data normalized by regression analysis and "percent of positive control" were then compared.

3. RESULTS

Reproducibility in the assay was based on 300 duplicates from 15 plates for the sera from uninfected non-vaccinated cattle, the coefficient of variation (CV) exceeded 25% an average of 7.1 ± 5.5 (SD) times per 40 duplicates on each plate. The high positive, low positive, and negative control sera, as well as the conjugate control did not exceed the upper and lower OD limits, as prescribed in the kit instructions, in any of the 15 plates. When evaluated by regression analysis of observed versus expected ODs for the serum controls within each plate, the average correlation coefficient for all plates was 0.996 (range of 0.990 - 1.000), indicating that the controls performed as expected. Comparison of the PP values for each serum control in all plates confirmed this high degree of reproducibility between plates: with the high positive control set at 100%, the low positive and the negative controls had mean PPs of 53.3% ± 4.1 and 5.0% ± 1.5, respectively. Visual inspection of plate data using sera from vaccinated and seropositive (by other tests) animals did not reveal any unexpected aberrations.

The cut-off for the sera from uninfected animals was determined for data normalized by regression analysis and by PP. Two samples had PPs of 173% and 89%, which were clearly outliers and were removed from the database. Of the remaining 598 samples, only 19 had PPs greater than 20%.

The mean plus 3 SD of the normalized ODs for these sera was 244 OD units. At this cut-off, the calculated specificity by was 98.3%. Similarly, when calculated using the mean PP + 3SD, the cut-off was 26% resulting in a specificity of 98.5% (one less animal was classified as "false positive" using the PP method than in the regression method of normalization).

The sera from vaccinated animals were evaluated to establish a cut-off reflective of antibody that might be detected as a result of vaccination. Of 272 sera, 6 exceeded the 100% PP of the positive control. Likewise, 13 (4.7%) of the animals exceeded PP of 70%, and 21 (7.7% of 272) exceeded a PP of 50%. These data represent a severe skew to the right in the frequency distribution of PP values. For this reason, it is not possible to use parametric statistics (Standard Deviation) that assume a normal distribution in determining the cutoff. Rather, 2 times the mean PP of all samples, including those exceeding PP of 100%, was 42.9%. If the PPs for the 6 samples exceeding 100% PP were eliminated, then the cut-off was calculated at 38.3%. If only those samples having PPs of <70% were included in the calculation, the cut-off would be 35.1%.

When these sera plus an additional 128 sera from vaccinated animals (n = 400) were evaluated using the monoclonal sera, the same skewing to the right occurred in the frequency distribution of PP values. Twice the mean of all of the PPs for these vaccinated animals resulted in a calculated cut-off of 34.4% as compared to 42.9% for the comparable samples using the polyclonal conjugate.

4. CONCLUSIONS

Reproducibility within the assay was acceptable with a minimum of variation between the plates. The only limitation was that CVs for the duplicates tended to be high with over 7% of the samples exceeding a CV of 25%. This occurred even when the mean of the duplicates exceeded 20 OD units.
Plate-to-plate variation as determined by reproducibility of the serum controls was minimal. All of the ODs for the controls fell within the upper and lower limits as indicated in the kit instructions. Also, the mean correlation coefficient of 0.996 for the regression of the controls versus their expected values was excellent. When the data normalized by the PP or regression method were compared, little difference was noted. The regression method has the advantage of normalizing all samples to a standard curve created by 4 samples rather than only the high-positive control as in the PP method. This indicated that the high positive control did not vary significantly between plates.

The cut-off (mean plus 3 times the SD) calculated by use of the panel of known uninfected and non-vaccinated animals, was 24 PP and 26 PP when data were normalized by the regression or the PP method, respectively. The calculated specificity at these cut-offs were 98.3% and 98.5%, respectively. The cut-off was only 14 PP when the convention of two times the mean of all samples was used. Using the mean + 3 x SD as the cut-off, the difference between data normalized by regression and PP resulted in a change in classification in only one of 598 animals. In contrast, attempts to calculate a cut-off for vaccinated animals that were RBT negative met with difficulties. Extreme skewing of the frequency distribution of PP values to the right precluded use of the mean plus three standard deviations convention in determining specificity. When twice the mean of all samples was used, the cut-off was 43%. This extreme difference (PP of 14 versus 43) between non-vaccinated/non-infected and vaccinated animals is probably due to vaccination of animals later than in their calfhood. We have no explanation of why the samples with high PPs are negative on Rose-Bengal; it was expected that the samples with high PP values would also have been positive on the RB test.

When the monoclonal and polyclonal conjugates were compared, the same skewing of the frequency distribution occurred for the monoclonal as for the polyclonal conjugate. Although the two times the mean PP value for the monoclonal conjugate was somewhat lower (34.4% versus 42.9%), the same trend occurred. This further suggests that the sera from vaccinated animals represented recent vaccinations for which antibody titers had not yet waned.

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REFERENCES

[1] CASAS OLASCOAGA, R., Algunos aspectos de Brucelosis Bovina, Banco de Seguros del Estado (ROU) (1964) 147-150.