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Interpretation of Spectrophotometric Absorbance Values to Define Results of Enzyme-Linked Immunosorbent Assays

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An enzyme-linked immunosorbent assay method was used to classify bovine serum as positive, negative, or doubtful for antibodies to *Brucella abortus*. Spectrophotometric data from assays of 64 serologically positive and 32 serologically negative bovine sera were analyzed statistically to define the range of spectrophotometric absorbance values which classify sera. Statistical analysis indicated that absorbance values <0.08 should be considered negative and values >0.14 should be considered positive, with intermediate values declared doubtful, and that the probability of erroneously classifying a positive serum as negative or a negative serum as positive is less than 0.005.

Serological assessment of *Brucella abortus* infection in cattle has been contingent upon results of agglutination and complement fixation assays (1). However, agreement among serological methods is influenced by vaccination status, subclasses, and affinity characteristics of immunoglobulins, immune complexes, and, possibly, other immunological factors (2, 3), making interpretation of serological results at best inconclusive.

Recently, immunoenzyme methods have been recognized as useful serological tools for determining epidemiological indices applicable to viral, bacterial, and parasitic infections (10, 11, 14, 15) and for the detection of toxins in foods for human consumption. However, the methods for interpreting results from enzyme-linked immunosorbent assays (ELISA) are without definition and are, therefore, completely arbitrary. Voller and Bidwell (15) expressed the results as spectrophotometric absorbance values (SAV) of unknown sera compared to the SAV of positive reference sera. To interpret their results, Byrd et al. (3) used differences in percent transmission of unknown and control sera expressed as extinction values. Saunders and Clinard (10) employed a signal-to-noise ratio, and Locarnini et al. (5) used a positive/negative ratio to express ELISA results. Visual interpretation of color, as used by Thoen et al. (13), apparently was satisfactory for interpreting enzyme immunoassays for detecting the A and M antigens in brucellae. However, Ruitenberg et al. (7) recognized the need for assessing data generated by an ELISA method by a different procedure if ELISA is to be used for large-scale testing purposes.

The objectives of this study were to define positive and negative ELISA results in terms of SAV and to demonstrate by statistical evaluation of ELISA results that SAV occur between positive and negative values and should be classified as doubtful.

MATERIALS AND METHODS

Positive sera. Sixty-four bovine sera, which were positive for *B. abortus* antibodies when tested by card, rivanol, standard tube agglutination, and complement fixation methods according to criteria previously described (F. C. Heck, J. B. Williams, R. P. Crawford, and A. I. Flowers, J. Hyg., in press), were designated positive sera. ELISA readings were determined on these sera by replicate testing at two different times, and the SAV were used to represent serologically positive absorbance values. All sera were tested at a 1:20 dilution in Tween-saline.

Negative sera. Thirty-two sera which were negative for *B. abortus* antibodies when tested by the card, rivanol, standard tube agglutination, and complement fixation methods according to criteria previously described (Heck et al., in press) were selected as serologically negative sera. All sera were tested by ELISA at a 1:20 dilution made in Tween-saline. ELISA readings were obtained on these sera after replicate tests were conducted at two different times, and the SAV were used to represent serologically negative absorbance values. Additionally, 170 sera which were obtained by sampling 10 cows negative for *B. abortus* for 17 consecutive days were tested by the ELISA method at two different dilutions, 1:3 and 1:20.

Test reproducibility. Twenty-five bovine sera which had SAV between 0.02 and 0.44 were tested in triplicate (replicate (R)1, R2, and R3) in a double-blind study to evaluate the reproducibility of the ELISA method. The data were analyzed by the paired *t*-test to test the hypothesis that the mean difference was zero.

Antigen. Soluble *B. abortus* antigens, lot 3 and lot 14, were kindly supplied by D. E. Pietz, USDA, Veterinary Services Laboratory, Ames, Iowa. Optimum

antigen concentrations for each lot were determined as described by Ruitenberg et al. (8). Absorbance values for R1 were obtained, using lot 3 antigen (5.3 mg of protein per ml) diluted 1:1,000, and absorbance values for R2 were obtained using lot 14 antigen (4.3 mg of protein per ml) diluted 1:750. Antigen was added (50 μl) to each well of a microtiter plate (Linbro-Titertek; Flow Laboratories, Hamden, Conn.) and allowed to dry overnight at 37°C.

Enzyme-conjugated anti-immunoglobulin. Lyophilized rabbit anti-bovine immunoglobulin G (Miles Laboratories, Inc., Elkhart, Ind.) was reconstituted in 5 ml of sterile water and precipitated with 70% ammonium sulfate (2). The precipitated protein was suspended to the original volume in 0.85% saline and dialyzed for 24 h against several changes of 0.01 M phosphate-buffered saline, pH 7.2. The antibody protein solution was lyophilized and stored at 4°C. Five milligrams of antibody protein was labeled with type VI horseradish peroxidase by the method of Nakane and Kawaoi (6). Optimum conjugate concentrations to insure low background and consistent absorbance values with a known standard positive serum were determined by titration by the methods of Ruitenberg et al. (8). Conjugate concentrations for determining absorbance values for R1 and R2 were 1:750 and 1:500, respectively.

ELISA test protocol. The ELISA was conducted as previously described (3) with the exception that in place of 5-amino-salicylic acid the indicator used in the substrate was 2,2′-azino-di-(3-ethyl-benz-thiazoline sulfonic acid) diluted in citric acid, pH 4.0. Color development was arrested by the addition of 0.1 ml of 0.01 M hydrofluoric acid. Absorbance values were read at 414 nm.

Statistical analysis. Absorbance values of serologically positive sera were recorded as positive replicate 1 (PR1) and PR2. Absorbance values from ELISA of serologically negative sera were recorded as negative replicate 1 (NR1) and NR2. The statistical parameters—means, standard deviations, standard errors of the mean, confidence limits, and *t*-tests—were calculated from ELISA data by the methods of Steel and Torrie (12).

RESULTS

Reproducibility. Data which were obtained by testing 25 sera in triplicate were analyzed by the paired *t*-test (4) to test the hypothesis that the mean difference was zero (Table 1). Means for the three possible differences, D12 (R1 minus R2), D31 (R3 minus R1), and D32 (R3 minus R2), were 0.0140, 0.0049, and 0.0079 (Table 2). The 0.99 confidence interval for all 3 means included zero (Table 2); therefore, the hypothesis that no difference exists among SAV on the 3 replicates is acceptable and the reproducibility of SAV is supported. The maximum absolute deviation of SAV was 0.1, which occurred in a sample with an R1 ELISA reading of 0.42. Differences between SAV >0.02 occurred only in positive samples with initial SAV ≥0.18 (Table 1).

TABLE 1. *Absorbance values and differences between absorbance values from 25 serum samples used to demonstrate test reproducibility*

Sample	Absorbance value (triplicate)			Difference between absorbance values		
	R1	R2	R3	D12	D31	D32
1	0.02	0.02	0.02	0.00	0.00	0.00
2	0.18	0.18	0.20	0.00	0.02	0.02
3	0.03	0.04	0.03	−0.01	0.00	−0.01
4	0.03	0.02	0.04	0.01	0.01	−0.02
5	0.02	0.03	0.04	−0.01	0.02	−0.01
6	0.04	0.04	0.04	0.00	0.00	0.00
7	0.40	0.40	0.40	0.00	0.00	0.00
8	0.34	0.26	0.34	0.08	0.00	0.08
9	0.02	0.04	0.04	−0.02	0.02	0.00
10	0.02	0.02	0.02	0.00	0.00	0.00
11	0.04	0.04	0.05	0.00	0.01	0.01
12	0.40	0.36	0.42	0.04	0.02	0.06
13	0.26	0.21	0.25	0.05	−0.01	0.04
14	0.31	0.31	0.37	0.00	0.06	0.06
15	0.32	0.34	0.30	−0.02	−0.02	−0.04
16	0.22	0.20	0.28	0.02	0.06	0.08
17	0.42	0.36	0.44	0.06	0.02	0.08
18	0.18	0.18	0.12	0.00	−0.06	−0.06
19	0.03	0.03	0.03	0.00	0.00	0.00
20	0.42	0.34	0.44	0.08	0.02	0.10
21	0.31	0.26	0.31	0.05	0.00	0.05
22	0.02	0.03	0.02	−0.01	0.00	−0.01
23	0.40	0.40	0.44	0.00	0.04	0.04
24	0.18	0.16	0.18	0.02	0.00	0.02
25	0.39	0.38	0.43	0.01	0.04	0.05

TABLE 2. *Confidence intervals for the mean differences between three absorbance values for randomly chosen sera*[a]

Sample	*n*	Mean difference	Standard error of the mean	Confidence interval (=0.01)	Data range
D12	25	0.0140	0.0058	0 to 0.03	−0.02, 0.08
D31	25	0.0100	0.0049	0 to 0.02	−0.06, 0.06
D32	25	0.0240	0.0079	0 to 0.05	−0.10, 0.06

[a] Absorbance values and differences between absorbance values are presented in Table 1.

Statistical analysis of serologically positive and negative sera. In Table 3 we present the results of a statistical analysis on the absorbance values from 64 positive and 32 negative sera. These same sera were tested at two different times with different lots of the same antigen preparation to yield replicates of the positive and negative samples. The confidence limits were calculated by using a one-tailed *t*-test at the 0.005 probability level.

The confidence limits calculated for the positive replicates indicate that the probability of an absorbance value for a positive sample being <0.11 for PR1 (Fig. 1) or <0.08 for PR2 (Fig. 2)

TABLE 3. *ELISA results from sera of known serological status*

Serological status	*n*	Mean	Standard deviation	One-tailed confidence limit (0.995)	Data range	Coefficient of variation
PR1	64	0.3655	0.0960	0.1101	0.05 to 0.52	26.26
PR2	64	0.2797	0.0764	0.0765	0.03 to 0.39	27.31
NR1	32	0.0506	0.0324	0.1397	0.00 to 0.15	64.03
NR2	32	0.0534	0.0198	0.1079	0.02 to 0.10	37.08

is <0.005. For negative replicates the confidence limits indicate that the probability of a SAV for a negative sample being >0.14 for NR-1 (Fig. 1) or >0.11 for NR2 (Fig. 2) is <0.005. For PR1 and NR1, a SAV >0.11 and <0.14 (Fig. 1) or for PR2 and NR2, a SAV >0.08 and <0.11 (Fig. 2) is considered doubtful. For serologically negative sera (i.e., NR1) we expect 95% of the population to have a SAV ≤0.11 and 0.5% of the population to have a SAV ≥0.14 and, therefore, to be erroneously classified as positive (Fig. 1), leaving 4.5% of the population classified as doubtful. For serologically negative sera (i.e., NR2), we expect 90% of the population to have a SAV ≤0.08 and 0.5% of the population to have a SAV ≥0.11 and be erroneously classified as positive. The remaining 9.5% of the population would be classified as doubtful (Fig. 2).

For serologically positive sera (i.e., PR1) we expect 99% of the population to have a SAV ≥0.14 and 0.5% of the population to have a SAV ≤0.11 and be erroneously classified as negative. The remaining 0.5% of the population would be classified as doubtful (Fig. 1). For serologically positive sera (i.e., PR2) we expect 98.5% of the population to have a SAV ≥0.11 and 0.5% of the population to have a SAV ≤0.08 and be erroneously classified as negative. The remaining 1.0% of the population would be classified as doubtful (Fig. 2).

Statistical analysis of ELISA results from serologically negative sera tested at 1:3 and 1:20 dilutions are presented in Table 4. The SAV on sera tested at a 1:3 dilution were generally higher than the SAV on sera tested at a 1:20 dilution. However, data from sera tested at a 1:20 dilution support the data in Table 1 regarding the values expected for serologically negative sera. Furthermore, the ELISA results on 170 sera from 10 cows negative for *B. abortus* support the results of the study of reproducibility shown in Table 2.

DISCUSSION

The nature of the ELISA method is such that there is a gradual change from negative to positive serological classification based on increasing

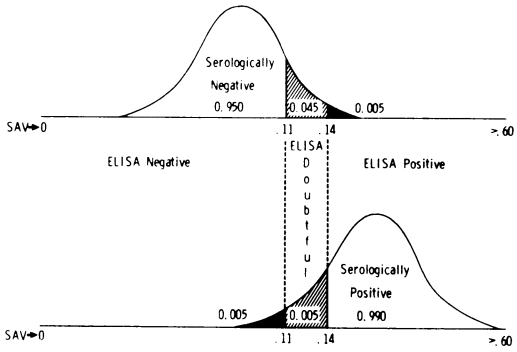

FIG. 1. *Proportional distribution of serologically positive and serologically negative sera, replicate 1, classified as ELISA-positive, -negative, and -doubtful.*

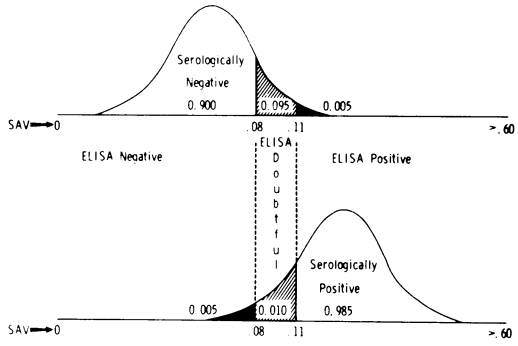

FIG. 2. *Proportional distribution of serologically positive and serologically negative sera, replicate 2, classified as ELISA-positive, -negative, and -doubtful.*

TABLE 4. *Analysis of ELISA test results from two different dilutions of sera collected for 17 consecutive days from each of 10 B. abortus-negative cattle*

Dilution	*n*	Mean	Standard deviation	CV[a]	Upper limit
1:3	170	0.0804	0.0331	41.17	0.1574
1:20	170	0.0269	0.0107	39.78	0.0518

[a] CV, Coefficient of variation, which expresses the standard error as a percentage of the mean, and is used to express relative variation between the two dilutions.

SAV. After statistical analysis of the SAV from serologically positive and negative sera, definition of serum as ELISA-positive, -negative, or -doubtful was achieved. Lower SAV are considered negative; higher SAV are considered positive. Sera for which the SAV fall into the intermediate range are considered doubtful.

The reliability of the ELISA procedure, that is, the ability to repeat absorbance values from tests conducted at different times, is good. This is confirmed by the fact that mean differences

expressed in Table 2 are not significantly different from zero. Therefore, any difference between mean absorbance values must be considered a real difference and not a difference due to experimental error.

The differences, represented in Table 3, between the means of the samples known to be serologically positive or negative are attributed in part to the different lots of antigen used to test the replicate samples. Although the means are different, the coefficients of variation of the two positive replicates are almost identical, which indicates the same relative variation in SAV. The coefficients of variation of the negative replicates are large as a result of the low absorbance values which are characteristic of negative sera. Small changes in SAV are reflected in large percent values at the low end of the negative scale.

Figures 1 and 2 show that SAV on serologically negative sera were <0.11 or <0.08 and, therefore, they should be considered ELISA-negative. Similarly, SAV on serologically positive sera were >0.11 and >0.14, respectively, and therefore they should be considered ELISA-positive. However, we chose to use conservative figures to define SAV as positive, negative, or doubtful. These figures were derived by combining data from Fig. 1 and 2 whereby a SAV <0.08 is considered negative and a SAV ≥0.14 is considered positive. Absorbance values >0.08 and <0.14 are considered doubtful.

Data in Table 4 indicate that the coefficients of variation are nearly the same, which implies that the relative variation between the samples is not different. ELISA results or SAV from sera tested at a 1:3 dilution yield a threefold increase in the mean and standard deviation when compared to ELISA results from sera tested at a 1:20 dilution. Thus, new criteria must be developed for defining positive, negative, or doubtful sera when changes in test conditions occur, because changes in antigen or antigen concentration, and dilution of antiserum or conjugate or both can effect the SAV. Therefore, these variables should be standardized by box titration before the criteria for defining positive or negative sera are established. The need for serological evaluation of sera at higher concentrations was indicated from our recent experience that early detection of serum antibodies from reactor cows was accomplished more frequently if sera were tested at a dilution <1:20.

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LITERATURE CITED

1. **Alton, G. G., L. M. Jones, and D. E. Pietz.** 1975. Laboratory techniques in brucellosis, 2nd ed. World Health Organization, Albany, N.Y.
2. **Beh, K. J.** 1974. Quantitative distribution of brucella antibody amongst immunoglobulin classes in vaccinated and infected cattle. Res. Vet. Sci. **17**:1-4.
3. **Byrd, J. W., F. C. Heck, and R. J. Hidalgo.** 1979. Evaluation of the enzyme-linked immunosorbent assay for detecting *Brucella abortus* antibodies. Am. J. Vet. Res. **40**:896-898.
4. **Eckschlager, K.** 1961. Errors, measurement and results in chemical analysis. Van Nostrand Reinhold Co., London.
5. **Locarnini, S. A., A. G. Coulepis, A. M. Stratton, J. Kaldor, and I. D. Gust.** 1979. Solid-phase enzyme-linked immunosorbent assay for detection of hepatitis A-specific immunoglobulin. J. Clin. Microbiol. **9**:459-465.
6. **Nakane, P. K., and A. Kawaoi.** 1974. Peroxidase-labeled antibody: a new method of conjugation. J. Histochem. Cytochem. **22**:1084-1091.
7. **Ruitenberg, E. J., B. J. M. Brosi, and P. A. Steerenberg.** 1976. Direct measurement of microplates and its application to enzyme-linked immunosorbent assay. J. Clin. Microbiol. **3**:541-542.
8. **Ruitenberg, E. J., P. A. Steerenberg, B. J. M. Brosi, and J. Buys.** 1976. Evaluation of the reliability of immunoenzymatic techniques for the serodiagnosis in *Trichinella spiralis* infections, p. 149-165. *In* G. Feldmann, P. Druet, J. Bigrion, and S. Avrameas (ed.), Immunoenzymatic techniques. Elsevier/North Holland Publishing Co., Amsterdam.
9. **Saunders, G. C.** 1977. Development and evaluation of an enzyme-labeled antibody test for the rapid detection of hog cholera antibodies. Am. J. Vet. Res. **38**:21-25.
10. **Saunders, G. C., and E. H. Clinard.** 1976. Rapid micromethod of screening for antibodies to disease agents using the indirect enzyme-labeled antibody test. J. Clin. Microbiol. **3**:604-608.
11. **Saunders, G. C., and M. E. Wilder.** 1974. Disease screening with enzyme-labeled antibodies. J. Infect. Dis. **129**:362-364.
12. **Steel, R. G., and J. H. Torrie.** 1960. Principles and procedures of statistics. McGraw-Hill Book Co., New York.
13. **Thoen, C. O., A. L. Armbrust, W. G. Eacret, R. Harrington, Jr., and G. M. Brown.** 1979. Use of an enzyme immunoassay test for characterizing the A and M antigens of *Brucella*. J. Clin. Microbiol. **9**:485-487.
14. **Voller, A., A. Bartlett, and D. E. Bidwell.** 1976. Enzyme immunoassays for parasitic diseases. Trans. R. Soc. Trop. Med. Hyg. **70**:98-106.
15. **Voller, A., and D. E. Bidwell.** 1976. Enzyme immunoassays for antibodies in measles, cytomegalovirus infections and after rubella vaccination. Br. J. Exp. Pathol. **57**:243-247.