Evaluation of Primary Binding Assays for Presumptive Serodiagnosis of Swine Brucellosis in Argentina

P. SILVA PAULO,1,* A. M. VIGLIOCCO,1 R. F. RAMONDINO,1 D. MARTICORENA,1 E. BISSI,1 G. BRIONES,1 C. GORCHS,1 D. GALL,2 AND K. NIELSEN2

Comision Nacional de Energia Atomico Centro Atomico Ezeiza, Ciudad de Buenos Aires, Argentina,1 and Canadian Food Inspection Agency, Animal Diseases Research Institute, Nepean, Ontario, Canada K2H 8P92

Received 29 November 1999/Returned for modification 10 March 2000/Accepted 17 May 2000

An indirect enzyme-linked immunosorbent assay (IELISA), a competitive ELISA (CELISA), and a fluorescence polarization assay (FPA) for the presumptive serological diagnosis of swine brucellosis were evaluated using two populations of swine sera: sera from brucellosis-free Canadian herds and sera from Argentina selected based on positive reactions in the buffered antigen plate agglutination test (BPAT) and the 2-mercaptoethanol (2-ME) test. In addition, sera from adult swine from which Brucella suis was isolated at least once for each farm of origin were evaluated. The IELISA, CELISA, and FPA specificity values were 99.9, 99.5, and 98.3%, respectively, and the IELISA, CELISA, and FPA sensitivity values relative to the BPAT and the 2-ME test were 98.9, 96.6, and 93.8%, respectively. Actual sensitivity was assessed by using 37 sera from individual pigs from which B. suis was cultured, and the values obtained were as follows: BPAT, 86.5%; 2-ME test, 81.1%; IELISA, 86.5%; CELISA, 78.5%; and FPA, 80.0%.

Brucella suis occurs naturally in the smooth phase. The smooth Brucella spp. share certain smooth lipopolysaccharide (SLPS) epitopes, resulting in extensive serological cross-reactions. For this reason, conventional serological tests for swine brucellosis use antigens from Brucella abortus rather than B. suis, as shown by extensive testing for swine brucellosis in the United States (6). Conventional serological tests (16) and indirect enzyme-linked immunosorbent assays (IELISAs) (4, 16) cannot identify animals infected with Yersinia enterocolitica O:9 because of epitopes shared with Brucella spp. (16), resulting in lower specificity values than assays such as the competitive ELISA (CELISA) and the fluorescence polarization assay (FPA), which are capable of distinguishing animals infected with cross-reacting microorganisms (8, 9, 15) or vaccinated (FPA), which are capable of distinguishing animals infected with cross-reacting microorganisms (8, 9, 15) or vaccinated

MATERIALS AND METHODS

Negative sera. Sera from Canadian pigs with no clinical or epidemiological evidence of brucellosis (B. suis has not been detected in Canada) were used to evaluate the cutoff values and specificities for the IELISA, CELISA (n = 952), and FPA (n = 866).

Positive sera. Sera with positive reactions in the BPAT and the 2-ME test were used to evaluate relative sensitivities for the IELISA, CELISA (n = 469), and FPA (n = 387). All sera that gave positive results were retested using the IELISA, CELISA, and FPA. Due to insufficient volume, 82 sera were not tested in the FPA.

Sera from B. suis culture-positive animals. One hundred sixty blood samples, as well as retropharyngeal and mandibular lymph nodes, were simultaneously collected from adult swine at the time of slaughter. Blood samples were allowed to clot and were centrifuged and then kept frozen until they were tested. The lymph nodes were cut into pieces, macerated with a blade, and inoculated directly onto Farrel’s medium. All plates were incubated at 37°C for 8 days. Colonies suspected of being Brucella were identified by macroscopic appearance, acriflavine testing, and oxidase and urease reactions and were biotyped using standard procedures (13) and phagotyped using Brucella phages (1). Thirty-seven positive samples were obtained.

Serological tests. The BPAT and the 2-ME test were performed according to standard procedures (19). The IELISA, CELISA, and FPA were performed as described previously (11–13).

Briefly, the IELISA and CELISA used B. abortus SLPS (12) immobilized on a polystyrene matrix as the antigen. The IELISA used a monoclonal antibody specific for a heavy-chain epitope of porcine IgG, labeled with horseradish peroxidase (12), as the detection reagent. The dilution of serum for this assay was 1:50. Optical density values were obtained after 10 min of incubation with the substrate-chromogen system, and the data were presented as a percentage of the strong positive control serum included in each plate (%P).

The CELISA used a monoclonal antibody specific for an epitope of B. abortus O-polysaccharide (12) as the competition reagent and a goat anti-mouse IgG (H- and L-chain-specific) affinity-purified antibody-enzyme conjugate (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) as the detection reagent. For this assay, the final serum dilution was 1:20. Optical density values were determined after 10 min of incubation with the substrate-chromogen, and the data were expressed as a percentage of inhibition (%I) relative to the optical density of the buffer control (no inhibition control).

The FPA used the O-polysaccharide prepared from B. abortus SLPS conjugated with fluorescein isothiocyanate as a tracer (13). Each porcine serum, diluted 1:25, was evaluated in a fluorescence polarization analyzer (FFM-1; Jolley Consulting and Research, Round Lake, Ill.) to obtain a background reading. Then tracer was added to each sample and mixed, and the mixture was incubated for at least 2 min. A second reading was obtained in millipolarization units (mP). The millipolarization units indicate the level of antibodies in the serum (the blank is automatically subtracted from each sample); a value higher than the cutoff value was considered positive.

* Corresponding author. Mailing address: Comision Nacional de Energia Atomico Centro Atomico Ezeiza, Av. del Libertador 8250, CP 1429, Ciudad de Buenos Aires, Argentina. Phone: 54-11-4379-8531. Fax: 54-11-4379-8540. E-mail: silvapau@cue.cnea.gov.ar.
Data analysis. The ability of the IELISA, CELISA, and FPA to discriminate positive sera from negative sera was evaluated using receiver operating characteristics (ROC) analysis (18). ROC software determines all possible sensitivity-specificity pairs with associated cutoffs, including the optimal values for each assay. Use of the ROC allows comparison of different tests without the problems of arbitrariness inherent in single calculations of sensitivity-specificity pairs (3). The optimal cutoff values were used to compare the tests. ROC analysis was also used to determine the area under the curve (AUC), a measure of test accuracy. For example, an AUC of 0.95 indicates that a randomly selected individual animal from a positive population will have a test result value greater than a randomly selected individual animal from a negative population 95% of the time.

RESULTS

The cutoff values for the IELISA, CELISA, and FPA were 26%P, 29%I, and 87.7 mP, respectively (Fig. 1). Using these cutoff values, the sensitivity values relative to the BPAT and the 2-ME test for the IELISA, CELISA, and FPA were 98.9, 96.6, and 93.8%, respectively. The specificity values for the IELISA, CELISA, and FPA were 99.9, 99.5, and 98.3%, respectively. B. suis was isolated from 37 of 160 slaughtered pigs tested. Twenty-eight isolates were typed as B. suis biovar 1 (atypical), and nine were B. suis biovar 1 (5). Three of 37 sera from positive-culture pigs gave negative serological reactions in all tests (Table 1). The actual sensitivity values, based on 37 sera from which B. suis had been isolated, for the BPAT, 2-ME test, IELISA, CELISA, and FPA were 86.5 (32 of 37), 81.1 (30 of 37), 86.5 (32 of 37), 78.5 (29 of 37), and 80.0% (28 of 35), respectively.

The AUCs for the IELISA, CELISA, and FPA were 0.995, 0.979, and 0.976, respectively. This shows that in more than 97% of cases an animal giving a result above the cutoff value was also used to determine the area under the curve (AUC), a measure of test accuracy. For example, an AUC of 0.95 indicates that a randomly selected individual animal from a positive population will have a test result value greater than a randomly selected individual animal from a negative population 95% of the time.

DISCUSSION

Conventional serological tests are not reliable for the presumptive diagnosis of porcine brucellosis in individual animals (16). The BPAT is prescribed by the Office International des Epizooties and is widely used as a screening test; however, its sensitivity and specificity are generally considered low (16). The complement fixation test is not considered suitable for individual animal testing, since swine complement interacts with guinea pig complement, reducing the sensitivity of the test (16). The 2-ME test is not included in the Office International des Epizooties manual. A general problem with all conventional tests and some primary binding assays is that they detect antibody resulting from exposure to cross-reacting organisms, such as Y. enterocolitica O:9, a problem in some areas (4, 21). This is demonstrated by data found at the Statens Veterinaere Serumlaboratorium, Copenhagen, Denmark, website (http://www.svs.dk/dk/ye-rapp.htm) showing that the complement fixation test does not distinguish between antibodies produced by Y. enterocolitica O:9 and antibodies produced by B. suis or B. abortus in 70% of the sera tested while the CELISA was positive in only 14% of the cases.

IELISAs have been developed, and while their sensitivities and specificities are higher than those of the conventional tests, they still detect antibody to cross-reacting microorganisms, such as Y. enterocolitica O:9 (4, 16). This explains why the relative sensitivity and actual sensitivity in this study surpassed those of other primary binding assays, as shown in another study (15).

The CELISA and FPA have been shown to decrease the frequency with which vaccinal and cross-reacting antibody is detected in cattle, with the FPA performing slightly better (11, 14). Since the parameters for these assays are similar when testing porcine sera, it would be reasonable to infer that interfering antibody may be eliminated in some cases, resulting in more specific assays. In a previous study the CELISA, correlated highly with combined BPAT and 2-ME test results (A. M. Vigliocco, D. Marticorena, R. Ramondino, P. Silva Paulo, C. Briones, E. Bisi, and K. Nielsen, Abstr. Int. Symp. Dis. Control

### Table 1. Comparison of assay results where 100% agreement was not shown

| Animal Status | Culture Status | BPAT (%) | 2-ME Test (%) | IELISA (%) | CELISA (%) | FPA (%) |
|---------------|---------------|----------|--------------|------------|------------|--------|
| 3 Positive    | Positive      | 0        | 71           | 64         | NT         |        |
| 18 Positive   | Positive      | 200      | 71           | 62         | 61.9       |        |
| 22 Positive   | Negative      | 0        | 1            | 25         | NT         |        |
| 26 Positive   | Positive      | 25       | 64           | 60         | 77.9       |        |
| 28 Positive   | Negative      | 0        | 26           | 6          | 78.2       |        |
| 29 Positive   | Positive      | 25       | 70           | 7          | 105.3      |        |
| 30 Positive   | Negative      | 0        | 2            | 2          | 75.2       |        |
| 31 Positive   | Positive      | 25       | 66           | 26         | 99.0       |        |
| 32 Positive   | Negative      | 0        | 13           | 3          | 80.8       |        |
| 33 Positive   | Positive      | 0        | 1            | 1          |            | 78.2   |
| 35 Positive   | Negative      | 0        | 1            | 7          | 81.8       |        |

* Cutoff values were as follows: 2-ME test, 1:25 dilution; IELISA, 26%P; CELISA, 29%I; FPA, 87.7 mP. NT, not tested.
21st Century, abstr IAEA-SM348/34p, p. 77, 1997). This is also the case in the current study, resulting in specificity and relative sensitivity values of 99.5 and 96.6% for the CELISA. Both the FPA and the CELISA gave very similar results, with the IELISA performing marginally better in this study, as in a previous study (15). In the FPA, the data may have been influenced by using freeze-dried sera for the negative population. Freeze-dried sera frequently do not completely dissolve when reconstituted, and sizeable complexes may remain, causing elevation of the millipolarization units for negative sera, thereby decreasing the assay sensitivity.

A problem with using conventional tests to validate new tests is that inaccuracies in the former are considered as failure of the latter. Thus, defined sera obtained from animals with proven infection would be ideal, but only small numbers of such sera were available for this study. In the current study, 37 sera from pigs from which B. suis was cultured were used to decide the actual sensitivity. Of the 37 sera, 3 were negative in all tests, 5 were negative on the BPAT and IELISA, 7 were negative on the 2-ME test and FPA (two fewer animals were tested due to insufficient serum), and 8 were negative on the CELISA (Table 1), resulting in low actual sensitivity values. This may be due to selection techniques, such as the use of pigs 2 to 3 months of age, which are susceptible to infection with B. suis but have very limited ability to produce antibody (16). There may also be a lack of antibody response in the very early stages of infection, or seronegative animals may have been infected without exhibiting any serological or clinical evidence of infection (21). The actual sensitivity values were close, due to the small sample set, and predicting which test would be more accurate would be difficult. In comparison, the actual sensitivity values obtained in a larger study (15) for the BPAT, IELISA, CELISA, and FPA were 77.1, 94.0, 90.8, and 93.5% with sera from 401 pigs infected with B. suis from Argentina, Chile, Mexico, and the United States. The small data set has shown that culture results in the current study may be biased due to insufficient numbers and selection of animals exhibiting clinical signs.

The relative sensitivity values of the IELISA, CELISA, and FPA in the current study were 98.9, 96.6, and 93.8%, respectively, indicating that the relative sensitivity values were as good as the actual sensitivities achieved in the larger study with culture-positive pigs (15). The specificity values in this study for the IELISA, CELISA, and FPA were 99.9, 99.5, and 98.3%, respectively, which was better than the specificity values achieved in the larger study (15) using 14,037 sera from a Canadian national survey performed in 1997. The specificity values for the IELISA, CELISA, and FPA in this larger study were 97.8, 96.6, and 97.2%, respectively. Since the BPAT and the 2-ME test are considered inaccurate in the diagnosis of porcine brucellosis, the estimates of sensitivity and specificity for the IELISA, CELISA, and FPA will be biased. The effect of this bias will be to inflate the relative sensitivity and specificity values for the IELISA, CELISA, and FPA, since they will be correlated with the BPAT and 2-ME test (3). This may explain why the relative sensitivity and specificity values for the IELISA, CELISA, and FPA are higher in this study than in the larger study.

For a new test for the presumptive diagnosis of brucellosis to be accepted, it would be required to perform as well as or better than the tests in use or to possess an advantageous attribute. The data reported for the diagnosis of B. suis in swine in this study show that the IELISA appeared to perform slightly better than the CELISA or FPA for sensitivity relative to the 2-ME test or the actual sensitivity based on culture results. However, the IELISA cannot differentiate antibody to cross-reacting microorganisms, such as Y. enterocolitica O:9, from antibody to B. abortus. Both the CELISA and the FPA have this capability. The ability to differentiate is important, since yersiniosis sometimes occurs in pigs (4). The CELISA is more complex and labor-intensive than the FPA, which is a very simple and inexpensive test compared with the IELISA and CELISA. It has the added advantage of adaptability to use outside the laboratory setting. The relative sensitivity and specificity obtained in this study exceed the actual sensitivity and specificity in a larger study, indicating that its performance is consistent with that shown in the larger study (15). The actual sensitivity obtained in the larger study (n = 401) was significantly higher than the actual sensitivity obtained in this study due to the small sampling size (n = 37).

ACKNOWLEDGMENTS

This project was supported in part by the National Agency for Promoting Science and Technology (Argentina) Project 08-04311 and by the Canadian Food Inspection Agency.

We acknowledge Norma Pereyra and Fernando Cane (Instituto de Porcicultura, Chanar Ladeado, Pcia de Santa Fe, Argentina) for the sera supplied.

REFERENCES

1. Alton, G. G., L. M. Jones, R. D. Angus, and J. M. Verger. 1988. Techniques for the brucellosis laboratory. p. 13–61. Institut National de la Recherche Agronomique, Paris, France.
2. Angus, R. D., and C. E. Barton. 1984. The production and evaluation of a buffered plate antigen for use in a presumptive test for brucellosis. Dev. Biol. Stand. 56:349–356.
3. Begg, C. B. 1987. Biases in the assessment of diagnostic tests. Stat. Med. 4:411–423.
4. Corbel, M. J. 1985. Recent advances in the study of Brucella antigens and their serological cross reactions. Vet. Bull. 55:927–941.
5. Corbel, M. J., E. L. Thoma, and C. Garcia Carrillo. 1984. Taxonomic studies on some atypical strains of Brucella suis (Br.). Vet. J. 140:33–43.
6. Deyoe, B. L. 1987. In A. D. Leman, B. Straw, R. D. Glock, W. L. Mengelin, R. H. C. Penny, and E. Scholl (ed.), Diseases of swine, 6th ed. Iowa State University Press, Ames.
7. Garcia Carrillo, C., V. Cedro, and L. De Benedetti. 1971. Evaluacion de tecnicas serologicas en cerdos con infeccio ´n reciente de Brucella suis. Revista de Investigaciones Agropecuarias, INTA, Republica Argentina, serie 4, vol. VIII, no. 4, p. 99–107.
8. Nielsen, K., K. W. Cheronenogrodzky, R. J. Duncan, and D. R. Bundle. 1989. Enzyme-linked immunosorbent assay for differentiation of the antibody response of cattle naturally infected with Brucella abortus or vaccinated with strain 19. Am. J. Vet. Res. 50:5–9.
9. Nielsen, K. 1990. The serological response of cattle immunized with Yersinia enterocolitica O:9 or O:16 to Yersinia and Brucella abortus antigens in bovine immunosera. Vet. Immunol. Immunopathol. 24:373–382.
10. Nielsen, K., D. Gall, W. A. Kelly, D. Henning, and M. Garcia. 1992. Enzyme immunoassay: application to diagnosis of bovine brucellosis. Agriculture Canada, Nepean, Canada.
11. Nielsen, K., L. Kelly, D. Gall, S. Balsevicius, P. Nicoletti, and W. Kelly, 1995. Improved competitive enzyme immunoassays for the diagnosis of bovine brucellosis. Vet. Immunol. Immunopathol. 46:285–291.
12. Nielsen, K., D. Gall, A. Kelly, A. Vigiloco, D. Henning, and M. Garcia. 1996. Immunosassay development—application to enzyme immunoassays for the diagnosis of brucellosis. Agriculture and Agri-Food Canada, Nepean, Canada.
13. Nielsen, K., D. Gall, M. Jolley, G. Leishman, S. Balsevicius, P. Smith, P. Nicoletti, and F. Thomas. 1996. Homogeneous fluorescence polarization assay for detection of antibody to Brucella abortus. J. Immunol. Methods 195:161–168.
14. Nielsen, K., D. Gall, M. Lin, C. Massangill, L. Samartino, B. Perez, M. Coats, S. Henager, A. Djerj, P. Nicoletti, and F. Thomas. 1998. Diagnosis of bovine brucellosis using a homogeneous fluorescence polarization assay. Vet. Immunol. Immunopathol. 66:321–329.
15. Nielsen, K., D. Gall, P. Smith, A. Vigiloco, B. Perez, L. Samartino, P. Nicoletti, A. Djerj, and F. Fearnth. 1999. Validation of the fluorescence polarization assay as a serological test for the presumptive diagnosis of porcine brucellosis. Vet. Microbiol. 68:245–253.
16. Office International des Epizooties. 1996. Manual of standards for diagnostic tests and vaccines, p. 471. Office International des Epizooties, F/Agri Stand.
17. Priadi, A., V. Casanah, R. G. Hirst, J. J. Emmins, J. van der Giessen, and M. Soeroso. 1986. Development of an enzyme-linked immunosorbent assay

Downloaded from http://cvi.asm.org on March 19, 2020 by guest
18. Schooijans, F., A. Zaleta, C. E. Depuydt, and F. H. Comhaire. 1995. MedCalc: a new computer program for medical statistics. Comput. Methods Programs Biomed. 48:257–262.
19. Servicio Nacional de Sanidad Animal. 1993. Procedure manual: serological diagnosis of bovine brucellosis. Servicio Nacional de Sanidad Animal. Buenos Aires, Argentina.
20. Thoen, C. O., M. P. Hopkins, A. L. Armbrust, R. D. Angus, and D. E. Pietz. 1980. Development of an enzyme-linked immunosorbent assay for detecting antibodies in sera of Brucella suis infected swine. Can. J. Comp. Med. 44:294–298.
21. Van Aert, A., P. Brion, P. Dekeyser, L. Uytterhaegen, R. J. Sijens, and A. Boeyé. 1984. A comparative study of ELISA and other methods for the detection of Brucella antibodies in bovine sera. Vet. Microbiol. 10:13–21.
22. Wrathall, A. E., E. S. Broughton, K. P. W. Gill, and G. P. Goldsmith. 1993. Serological reactions to Brucella species in British pigs. Vet. Rec. 132:449–454.