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Comparison of Enzyme-Linked Immunosorbent Assay and Complement Fixation Test for the Detection of Specific Antibody in Cattle Vaccinated and Challenged with *Brucella abortus*

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An enzyme-linked immunosorbent assay (ELISA) and a complement fixation test (CFT) were applied to sera collected regularly from 60 cattle challenged with *Brucella abortus* strain 544. Of the 60 cattle, 48 were vaccinated with either *B. abortus* strain 19 or *B. abortus* strain 45/20 as calves or adults. The remaining 12 cattle were not vaccinated. Of the 27 sera from cattle found to be infected at slaughter, 9 showed aberrant reactions to the CFT in that a positive titer after challenge was delayed or transient. The performance of the ELISA in these nine cattle with aberrant reactions and the other cattle in the trial was as follows. After vaccination with strain 19 or strain 45/20, the number of weeks at which the ELISA was positive was significantly greater ($P < 0.001$) than that for the CFT. The strain 19 vaccine also induced positive responses to the CFT and the ELISA for a significantly longer period ($P < 0.001$) than did the strain 45/20 vaccine. For cattle with aberrant reactions, the number of weeks after challenge when the ELISA was positive was significantly greater ($P < 0.001$) than that for the CFT. For noninfected cattle, the average number of weeks after challenge when the CFT was negative (35 of 40) was higher than that for the ELISA (18 of 40).

In the control and eradication of bovine brucellosis, the primary objective of serological tests is to detect infected animals that might spread disease. Serious problems in the interpretation of these tests arise particularly when cattle may have been vaccinated with either live *Brucella abortus* strain 19 or killed *B. abortus* strain 45/20. Nevertheless, when the sensitivity and specificity of the available serological tests are considered, there is general agreement that, although the complement fixation test (CFT) is superior to other tests for the detection of this disease (2, 12), it does have limitations. First, the CFT often shows anticomplementary reactions as well as prozones where immunoglobulin G2 (IgG2) antibodies to *B. abortus* block IgG1 and IgM antibodies (11). Second, some infected cattle may show aberrant serological reactions and are not detected (7, 15). Third, maternally infected cattle may be difficult to differentiate from those recently vaccinated with either of the above vaccines (8, 16). Finally, there are variations in the test due to differences in technique (1).

The enzyme-linked immunosorbent assay (ELISA) (9) has proven to be a valuable technique that provides high sensitivity in a wide variety of serological assays. In this study, the CFT and the ELISA were compared in their reactions with serum from cattle whose history in relation to exposure to *Brucella* antigens was known. The cattle in this study were part of a large field experiment in which vaccinated cattle were challenged with virulent *B. abortus* 544 and then cultured for *B. abortus* at slaughter. The clinical results of challenge (see Table 1) have been described elsewhere (17).

MATERIALS AND METHODS

Experimental cattle. Sixty female calves, 3 to 6 months of age, from a brucellosis-free herd were divided into three groups. Group 1 (24 cattle) was vaccinated with the standard dose of *B. abortus* strain 19 (batch no. 320-1; Commonwealth Serum Laboratories, Melbourne, Victoria, Australia); group 2 (24 cattle) was vaccinated with two doses of strain 45/20 (batch no. V059; Philips Duphar Pty. Ltd., North Sydney, New South Wales, Australia) 6 weeks apart; and group 3 (12 cattle) was not vaccinated. Half of groups 1 and 2 were vaccinated as calves at 3 to 6 months of age, and half were vaccinated at 14 to 16 months of age. All cattle were mated at 14 to 16 months of age and examined rectally for pregnancy approximately 3 months after mating. At this time, the 46 pregnant cattle were challenged via the conjunctival route with 15×10^6 viable organisms of strain 544, and 14 nonpregnant cattle received 15×10^7 viable organisms of strain 544. They were studied for approximately 50 weeks after challenge (26 to 36 weeks after parturition) and then slaughtered. Samples from the cranial and caudal quarters of each udder; from the kidneys, liver, spleen, and uterus; and from the submaxillary, retropharyngeal, mesenteric, internal iliac, and supramammary lymph nodes were collected aseptically from each animal for culture. The method of culture was that described by Alton et al. (1).

Serology. Blood samples were collected from all animals 1 week before the date of vaccination and weekly thereafter for 8 weeks. The interval between sampling was then increased to 1 month and continued until challenge. Blood samples were obtained 1 week before challenge and weekly thereafter for 40 weeks until slaughter. All sera were subjected to the CFT and the ELISA, and a total of 3,500 sera were compared. The ELISA was performed with a commercial preparation of rabbit anti-bovine IgG conjugated to horseradish peroxidase (Miles Biochemicals, Elkhart, Ind.). The procedure was as follows. Polystyrene flat-bottomed microtiter trays (Flow Laboratories, Inc., McLean, Va.) were coated with *B. abortus* lipopolysaccharide (kindly supplied by P. Plackett, Commonwealth Scientific and Industrial Research Organization, Parkville, Melbourne, Australia) alkali-treated antigen (14) (2 μg/ml in phosphate-buffered saline, pH 7.3) by incubation with 200 μl per well overnight at 4°C. The coating solution was removed, and the trays were washed three times at room temperature with phosphate-buffered saline containing Tween 20. A 100-μl sample of an appropriate dilution of test serum (see below)

TABLE 1. Clinical, bacteriological, and serological results of challenge of cattle with virulent strain 544 after vaccination as calves or adults with strains 19 and 45/20

Group no.[a]	No. of cattle		No. of abortions or neonatal deaths due to challenge	No. of infected cattle[b]		
	Total	Pregnant		Total	With normal reactions to CFT	With aberrant reactions to CFT
1 (19 vaccinated)						
Calf	12	7	1	4	1	3
Adult	12	11	0	1	0	1
2 (45/20 vaccinated)						
Calf	12	10[c]	3	3 (2)[d]	3	2
Adult	12	10[c]	3	6 (2)	5	3
3 (Control)	12	8	6	6 (3)	9	0

[a] Calves were vaccinated at 3 to 6 months of age with strain 19 or with two doses of strain 45/20 6 weeks apart; adults were vaccinated at 14 to 16 months of age with strain 19 or with two doses of strain 45/20 6 weeks apart.
[b] Found to be infected by bacteriolgical examination at slaughter.
[c] One died.
[d] Numbers in parentheses represent numbers of cattle not pregnant during the trial.

was then added (in duplicate), and the trays were incubated at 37°C for 30 min. After three washes with distilled water containing 0.05% (vol/vol) Tween 20, a 100-μl sample of appropriately diluted conjugate (see below) was added and incubated at 37°C for 30 min. The washing procedure was repeated. A 100-μl sample of substrate-chromogen solution (10-ml sample of 5-aminosalicyclic acid [pH 6.0, 0.8 mg/ml] plus 0.2 ml of 0.1 M H_2O_2) was added, and the trays were incubated at room temperature for 25 min. The optical density of the solution in each well was read on a Multiskan spectrophotometer at a wavelength of 450 nm. Controls on each tray included a positive and a negative control serum.

To determine a cutoff point, we tested sera from 30 cattle in the control group which were negative by the Rose Bengal test and with no titer by the CFT, the serum agglutination test, or the indirect hemolysis test. The mean optical density of these sera was calculated as 0.19. The cutoff point was determined as the mean plus 3 standard deviations or 0.28. This optical density value was used to signify the lowest level at which *Brucella*-specific IgG was considered to be present. By these criteria, all 30 sera were negative by the ELISA.

The effect of the dilution of serum and conjugate on the reactivity of the ELISA was determined by a two-way titration of conjugate against positive and negative serum. The optimum value which allowed maximum use of materials without compromising the sensitivity of the test was obtained at a serum dilution of 1/250 and a conjugate dilution of 1/800.

The CFT was performed by the Australian standard method (3). Complement fixation at a dilution of 3 $\log_2$ (1:8), the level recommended by the Australian Bureau of Animal Health (4), was regarded as a positive reaction.

Serum samples were titrated 1:4 to 1:128, well beyond the point of significance, in the CFT. Titers determined by the CFT were expressed as $\log_2$ of the reciprocal of the last dilution at which a positive reaction occurred.

Serological classification of animals. For the purposes of this study, cattle found to be infected at parturition or necropsy were classified by their responses to the CFT as normally or aberrantly reacting animals. Infected cattle showing positive titers by the CFT within 8 weeks of challenge and which maintained these titers until slaughter were classified as normal. Infected animals that did not show positive titers by the CFT until 8 to 26 weeks after challenge and which did not maintain these titers until slaughter were classified as aberrant reactors. The serological responses of cattle that were challenged but in which no infection was found at slaughter were also considered for this paper.

RESULTS

Of the 60 cattle in the trial, 33 showed no infection with strain 544 at slaughter or death. Of these 33 animals, 26 were pregnant. Two of these 26 animals died early in the trial of unrelated causes, 22 had healthy calves (between 18 and 25 weeks after challenge), and the remaining 2 had stillborn calves (26 weeks after challenge). No infection was found in the tissues of these two stillborn calves or in the tissues or milk of the dams.

Of the 27 cattle shown to be infected with strain 544 at slaughter, 18 were normal reactors to the CFT, whereas the other 9 cattle, all of which but one yielded *B. abortus* from udder tissue or the supramammary lymph node or both, were aberrant reactors (as defined above); the remaining animal, animal 1, yielded *B. abortus* from the submaxillary lymph nodes. All of these nine aberrant reactors and 9 of the 18 animals showing normal responses had previously been vaccinated with either strain 19 or strain 45/20 (Table 1). Significantly more live calves were born to the aberrant reactors than to the normal reactors (Fishers exact probability, 0.004; $P < 0.01$).

For the animals vaccinated, the ELISA and the CFT were assessed by comparing the number of weeks after vaccination that each test was positive. The ELISA was positive for a significantly longer period ($P < 0.001$) than was the CFT, and on an average there was an 8-week difference. The ELISA was also positive in animals vaccinated as adults for a significantly longer period ($P < 0.001$) than in those vaccinated as calves, and this applied for both the strain 19 and the strain 45/20 vaccinees (Table 2). Nevertheless, a difference between the two vaccines was evident, as strain 19 induced positive responses by both the CFT and the ELISA for a significantly longer period ($P < 0.001$) than did the strain 45/20 vaccine (Table 2).

The progressive titers to the CFT and the level of specific IgG detected by the ELISA for the nine cattle classed as aberrant reactors are shown in Fig. 1.

For these aberrant reactors, the mean number of weeks after challenge when the test was positive was significantly higher ($P < 0.001$) by the ELISA than by the CFT. Indi-

TABLE 2. Percentage of weeks positive to the CFT and ELISA after vaccination as calves or adults with strain 19 or 45/20

Group no. and strain	% of weeks positive by:		No. of weeks after vaccination and before challenge
	CFT	ELISA	
1 (Calfhood vaccinees)			
19	24	44	58
45/20	2	15	47
2 (Adult vaccinees)			
19	75	94	22
45/20	5	52	22

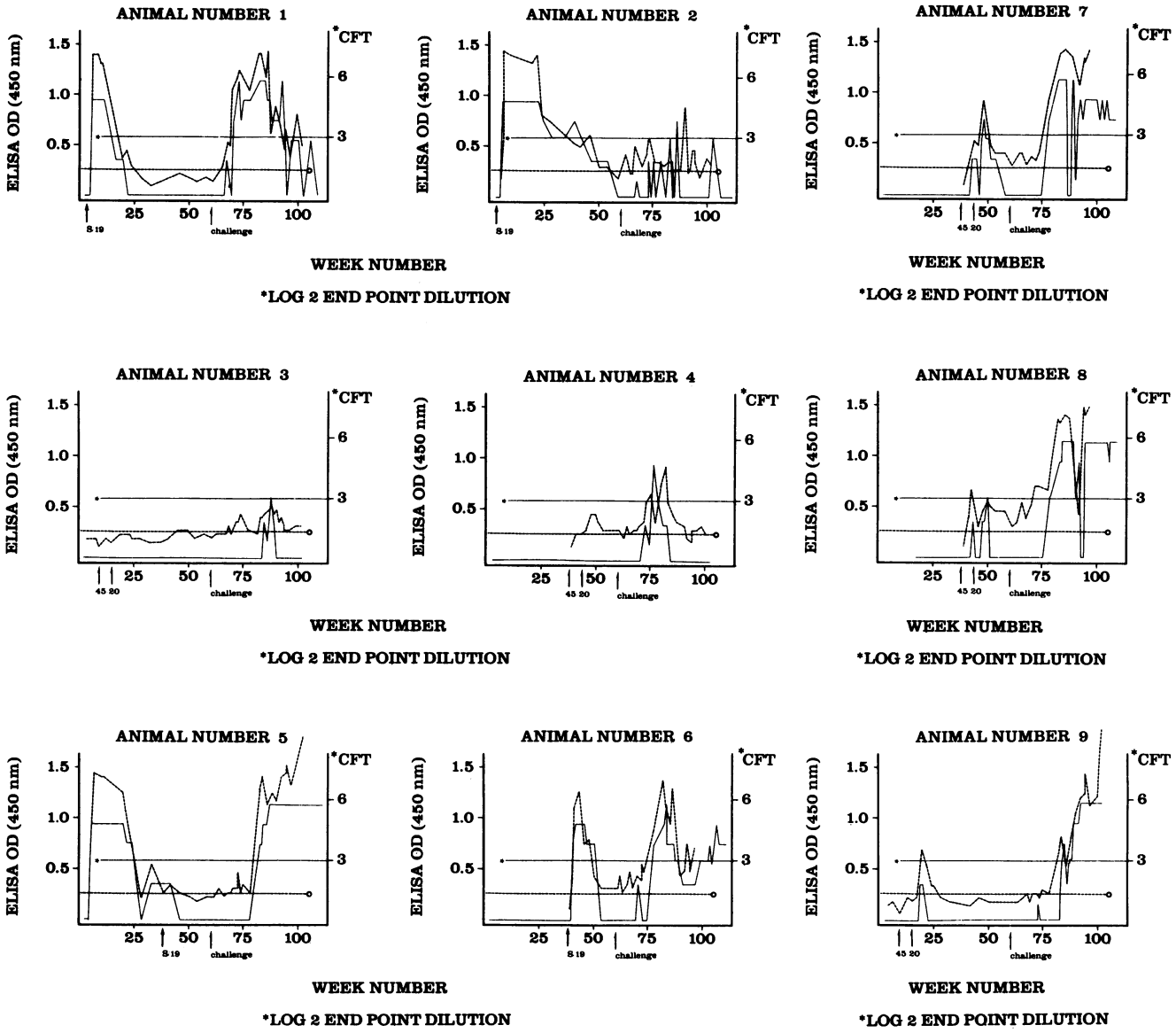

FIG. 1. Antibody levels in the sera of nine infected cattle after vaccination and challenge as measured by ELISA (– – –) and CFT (——). The horizontal dashed line divides the positive values from the negative values for the ELISA, and the horizontal continuous line divides the positive values from the negative values for the CFT.

vidual responses varied, however, as the ELISA was still positive at the time of challenge in three of the cattle classified as aberrant reactors (animals 6, 7, and 8), whereas in the remaining six cattle, the ELISA became positive 4 to 22 weeks earlier than the CFT (Table 3). Three of the nine cattle (animals 2, 3, and 4) had positive titers by the CFT for only 1 to 3 weeks of the 40-week postchallenge period, but these three were positive by the ELISA for 24, 28, and 33 weeks, respectively, of this period. Animal 6 was positive by the ELISA for the entire postchallenge period and positive by the CFT for only 12 weeks of the 40-week postchallenge period. Five of the nine cattle had negative titers by the CFT at slaughter, but all were positive by the ELISA (Table 3).

In the postchallenge period, the normal reactors showed little difference in the number of weeks positive by the two tests, but for the noninfected cattle, the average number of weeks (over all treatments) in the postchallenge period when the tests were negative was higher for the CFT (35 of 40 weeks) than for the ELISA test (18 of 40 weeks). There was no significant difference between the CFT and the ELISA for the three noninfected control cattle, but for other treatment groups the number of weeks when the test was negative postchallenge was always significantly higher ($P < 0.001$) by the CFT than by the ELISA.

DISCUSSION

In all of the cattle vaccinated with strain 19, high levels of *Brucella*-specific antibody were present within 1 to 2 weeks of vaccination and, although there was some agreement between the results of the ELISA and the CFT, the ELISA was positive for a significantly longer period after vaccination than was the CFT. If the ELISA were used for the detection of infected animals under field conditions, many false-positive reactions, which would lower the specificity of the test, could be expected (6).

TABLE 3. Time before CFT and ELISA became positive and serology at slaughter of nine infected cattle which showed aberrant serological reactions to the CFT

Animal no.	Vaccination history		Calf produced	Incubation period[a]		Reaction at slaughter by:	
	Age	Strain		CFT	ELISA	CFT	ELISA
1	Calf	S19	Healthy	8	4	−	+
2	Calf	S19	Healthy	24	2	−	+
3	Calf	45/20	Weak	26	8	−	+
4	Adult	45/20	Healthy	12	4	−	+
5	Calf	S19	Healthy	20	7	+	+
6	Adult	S19	Healthy	16	+[b]	−	+
7	Adult	45/20	Weak	16	+[b]	+	+
8	Adult	45/20	Healthy	16	b	+	+
9	Calf	45/20	None	21	7	+	+

[a] Weeks after challenge before positive titer.
[b] +, Positive at the time of challenge.

Brucella-specific antibody also developed after vaccination with the nonagglutinogenic rough strain 45/20, but the response to both tests was much weaker and the titers decreased markedly within 12 weeks of vaccination.

The cattle classified as normal reactors showed positive responses by the CFT within a few weeks of challenge and maintained them through slaughter; most of these aborted. In contrast, for the nine cattle classed as aberrant reactors by the CFT, several weeks or months elapsed after challenge before significant titers to this test were present. Many of these cattle had healthy calves, indicating that for most of the period after challenge, many of the cattle classified as aberrant reactors to the CFT would be both clinically and serologically indistinguishable from uninfected cattle in an eradication program based on the CFT alone. In all of these cattle the ELISA became positive earlier and lasted longer than the positive titers by the CFT. Nevertheless, the general trend of the ELISA, particularly in cattle classed as normal reactors, closely followed that of the CFT. In contrast, there was a greater number of positive reactions that occurred in noninfected cattle by the ELISA than by the CFT. Hence, it is suggested that in the early stages of an eradication program, when cattle are usually vaccinated to protect against abortion, the ELISA would be of little value in correctly identifying only infected cattle.

The greater sensitivity of the ELISA in this and other studies may be due to a characteristic secondary response that occurred as a result of the previous vaccination with the smooth strain 19 or the rough strain 45/20 (10, 13). Nevertheless, other reports have indicated that under certain circumstances, the secondary antibody response fails to occur (5, 18). It is possible that such a failure occurred in the aberrant reactor cattle in this study, as all of these cattle had been previously vaccinated with either strain 19 or strain 45/20 (Fishers exact test, 0.013; $P < 0.05$). Whatever the reason for the failure of the secondary antibody response in these cattle, they were all found to be infected, and all but one were capable of shedding the disease. Thus, particularly in the latter stages of an eradication campaign, detection of these aberrant reactors still remains a problem. Since other reports have also indicated a greater sensitivity of the ELISA when compared with the more conventional serological methods (10), it seems reasonable to suggest that in the latter stages of an eradication program, when vaccination has ceased, the ELISA may be useful as a screening or supplementary test to identify suspect animals that fail to respond by the CFT.

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