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Indirect Hemagglutination Employing Enterobacterial Common Antigen and *Yersinia* Somatic Antigen: a Technique to Differentiate Brucellosis from Infections Involving Cross-Reacting *Yersinia enterocolitica*

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The existence of enterobacterial common antigen in *Yersinia enterocolitica* and its absence in *Brucella abortus* were utilized in an attempt to provide a method to distinguish *Brucella* infections from infections with cross-reacting *Yersinia*. The indirect hemagglutination test was employed for this purpose. In experimental laboratory animals, the presence of anti-enterobacterial common antigen was found to be indicative of prior exposure to *Y. enterocolitica* rather than *B. abortus*. In cattle, however, low titers of anti-enterobacterial common antigen were present in all animals. It was observed that anti-enterobacterial common antigen titers either equaled or exceeded anti-*Yersinia* O titers in *Yersinia*-exposed animals, whereas in animals infected with *B. abortus* the anti-*Yersinia* O titer generally exceeded the anti-enterobacterial common antigen titer.

Brucella abortus and *Yersinia enterocolitica* serotype O9 possess a common O antigen (1, 3), as a result of which they show significant serological cross-reactivity. This cross-reactivity may cause confusion in the serodiagnosis and differentiation of brucellosis and yersiniosis. Several techniques have therefore been devised in an attempt to provide unambiguous serological identification of *Brucella*- and *Yersinia*-infected animals. These include quantitative slide agglutination (3), electroimmunoassay (4), enzyme-linked immunosorbent assay technique (B. Hurvell, E. Thal, H. E. Carlsson, and A. A. Lindberg, Proc. 20th World Vet. Congr., Thessaloniki, p. 787, 1975), and the modified tube and microagglutination tests (8; K. R. Mittal and I. R. Tizard, Res. Vet. Sci., in press; K. R. Mittal and I. R. Tizard, submitted for publication).

In spite of this serological cross-reactivity, *Y. enterocolitica* and *B. abortus* are unrelated. *Y. enterocolitica* is a member of the *Enterobacteriaceae*, whereas *B. abortus* is not. A characteristic feature of the enterobacteria is that they all possess enterobacterial common antigen (ECA) (6). This is a complex polysaccharide, probably a polymer of mannosaminuronic acid (6). ECA is a relatively powerful antigen, and antibodies to ECA are produced in animals infected with enterobacteria (6), including *Y. enterocolitica* (5). ECA is absent from *B. abortus* (6), and it was therefore anticipated that a rise in serum anti-ECA would be a feature of yersiniosis but not of brucellosis. We have therefore studied the levels of anti-ECA in experimental animals and cattle infected with either *B. abortus* or *Y. enterocolitica* O9 by using an indirect hemagglutination test. We have compared the results obtained by this technique with those obtained by the standard tube agglutination test (STA) and with indirect hemagglutination by using *Yersinia* somatic antigen. We also report on the use of these techniques in a survey of bovine sera submitted for brucellosis serology.

MATERIALS AND METHODS

Bacteria. Two smooth strains of *B. abortus* (S19 and 2308) were obtained from the stock culture collection of this laboratory. The strain of *Y. enterocolitica* O9 (strain 578) was kindly provided by S. Toma of the Ontario Ministry of Health, Toronto.

Animals and antiserum production. (i) Laboratory animals. Forty 400-g male guinea pigs were used. Ten received 1.0 ml of standard *B. abortus* strain 19 vaccine subcutaneously; 10 received 0.5 ml of *B. abortus* strain 2308 in Trypticase soy broth containing 10^5 colony-forming units (CFU) per ml; 10 received 0.5 ml of heat-killed standard *Brucella* tube test antigen (Agriculture Canada, Ottawa). The remainder received 0.5 ml of a broth culture of *Y. enterocolitica* O9 strain 578 containing 10^5 CFU/ml. The guinea pigs were bled 2 months postinfection, and the serum from each group was pooled.

A total of 150 outbred male white Swiss mice weighing 18 to 20 g were divided into three groups of 50

animals each. The animals in each group received 0.2 ml of either *B. abortus* strain 19, *B. abortus* strain 2308, or *Y. enterocolitica* O9 strain 578 subcutaneously as described above.

Two rabbits each received six intravenous inoculations of a heat-killed culture of *B. abortus* strain 19 at weekly intervals. Two other rabbits received a similar culture of heat-killed *Y. enterocolitica* O9. The rabbits were bled by cardiac puncture 7 days after the last injection. The serum was separated and stored at −20°C until used.

(ii) Calves. Eight castrated Holstein calves 3 months of age were each inoculated with 3 ml of standard live *B. abortus* strain 19 vaccine in the neck region by the subcutaneous route. Blood samples were taken at weekly intervals thereafter. Two similar calves each received an inoculation of 1 ml of a 24-h culture of *Y. enterocolitica* O9 and were bled at weekly intervals thereafter. All calves were bled 30 days after infection, and their serum was stored at −20°C until used.

(iii) Antigen production. *Yersinia* ECA and O antigen were extracted from *Y. enterocolitica* O9 (578) by the method of Suzuki et al. (9). *Y. enterocolitica* was grown on Trypticase soy agar in Roux flasks for 18 h at 37°C. The organisms were harvested by shaking with sterile glass beads in phosphate-buffered saline, pH 7.3. The resulting suspension was heated at 100°C for 1 h, and the supernatant fluid was harvested by centrifugation at 24,000 × *g*.

Ethanol was added to this fluid to yield a final concentration of 85%. After incubation at room temperature for 18 h, the suspension was centrifuged at 24,000 × *g*, the supernatant was decanted, and both ethanol-soluble and -insoluble fractions were evaporated to dryness in open petri dishes in an incubator at 37°C. The powders obtained were scraped off the glass and redissolved to a concentration of 1 mg/ml in either distilled water or phosphate-buffered saline. The ethanol-soluble fraction was considered to contain ECA, whereas the O antigen was ethanol insoluble.

Serological techniques. (i) IHA. Sheep erythrocytes were washed three times in phosphate-buffered saline and suspended to 2.5% in antigen solution. After incubation at 37°C for 1 h, the erythrocytes were again washed three times to remove unbound antigen. These treated erythrocytes were suspended to 0.5% concentration in phosphate-buffered saline. The indirect hemagglutination (IHA) test was performed in 0.025-ml volumes in microtiter plates (model I.S. MRC 96; Linbro Chemical Co., New Haven, Conn.). Tests were read after overnight incubation at 37°C.

(ii) STA tests. *Brucella* agglutinins were titrated by the standard methods of Morgan (7). *Yersinia* H and OH agglutination tests were performed by the methods of Mittal and Tizard (in press). (H agglutination is detected by performing a rapid agglutination test on formolized organisms at high temperature, and the test is read after flagellar agglutination but before significant somatic agglutination accurs. OH agglutination, in contrast, is measured by using formolized organisms incubated with antiserum for 24 to 48 h. The resulting agglutination involves both flagellar and somatic antigens.)

(iii) Microplate agglutination tests. Antigens for the microplate agglutination tests were made by staining organisms with 2:3:5 triphenyltetrazolium chloride (Difco Laboratories) by the method of Alton et al. (2). After staining, the bacterial suspensions were incubated at 100°C for 1 h to provide O antigens. The test was performed on microtiter plates. Twofold serum dilutions were employed, commencing at 1:10 in 0.025-ml volumes. Since the stained organisms settled rapidly, reproducible endpoints were observed after overnight incubation at 37°C after the addition of 0.025 ml of the antigen suspension to each well.

Bovine serum samples. Twenty serum samples were obtained from a *Brucella*-free herd of cattle; 70 samples were also obtained from those submitted for routine *Brucella* serology by the Brucellosis Laboratory, Agriculture Canada, Guelph, and were kindly made available by B. Kingscote.

RESULTS

Laboratory animals. Only those animals inoculated with *Y. enterocolitica* developed antibodies to ECA, as shown by IHA (Table 1). In contrast, all animals inoculated with either *B. abortus* or *Y. enterocolitica* O9 developed both direct agglutinins to *B. abortus* and indirect hemagglutinins to *Y. enterocolitica* O9 somatic antigen. It was not found possible to identify the *Yersinia*-infected animals and distinguish them from *Brucella*-infected laboratory animals by using only direct agglutination or IHA with *Yersinia* O antigen.

TABLE 1. *Antibody responses against Brucella and Yersinia antigens in pooled serum samples of mice, guinea pigs, and rabbits exposed to B. abortus and Y. enterocolitica O9*

Animal	STA *Brucella* antibody titer	IHA technique antibody titers against *Yersinia* antigens	
		O	ECA
Guinea pig anti-live *Y. enterolitica* O9	320	40	40
Rabbit anti-heat-killed *Y. enterolitica* O9	40	20	40
Mouse anti-live *Y. enterolitica* O9	80	80	80
Guinea pig anti-live *B. abortus* 2308	20,480	640	0
Guinea pig anti-live *B. abortus* S19	640	40	0
Guinea pig anti-heat-killed *B. abortus* tube test antigen	10,240	320	0
Rabbit anti-heat-killed *B. abortus* S19	10,240	640	0
Mouse anti-live *B. abortus* 2308	160	40	0
Mouse anti-live *B. abortus* S19	640	20	0
Mouse anti-heat-killed *B. abortus* tube test antigen	2,560	640	0

Experimentally infected calves. In two calves inoculated with *Y. enterocolitica* O9, the IHA titers to ECA were both 1:1,280 (Table 2). However, in the *B. abortus*-infected calves, IHA titers to ECA were also present and ranged from negative to 1:80 (geometric mean titer, 1:17). This difference was statistically significant ($P \geq 0.01$). All inoculated calves were positive for antibodies to *Brucella* when tested by STA and for antibodies to *Yersinia* O antigen when tested by IHA. In the case of the *Brucella*-infected calves, it was observed that the *Yersinia* O titers always exceed the ECA titer, whereas in the case of the *Yersinia*-infected calves the reverse occurred.

Bovine serums. Ninety bovine serum samples were tested. All possessed antibodies to ECA. Of these samples, 42 were considered to be negative for brucellosis in that they had STA titers of 1:40 (Fig. 1). Of these negative samples, 11 had ECA IHA titers greater than *Yersinia* O IHA titers, and 30 had identical ECA and *Yersinia* O titers. One animal had an ECA IHA titer of 1:10 and a *Yersinia* O IHA titer of 1:40. Thirty-seven samples were considered to be positive for brucellosis in that they had *Brucella* STA titers of 1:160. In seven of these positive samples, the ECA IHA titer was equal to the *Yersinia* O IHA titer. In the remaining 30 *Brucella*-positive samples, the *Yersinia* O exceeded the ECA IHA titer. Eleven bovine serum samples had a *Brucella* STA titer of 1:80. This titer is considered to be suspicious. Five of these samples had identical ECA and *Yersinia* O IHA titers, whereas five had ECA titers which exceeded the *Yersinia* O titer and one animal had a *Yersinia* O titer of 1:320 and an ECA titer of 1:40.

TABLE 2. *Antibody response in calves experimentally infected with either Y. enterocolitica O9 or B. abortus S19*

Calf infected with:	*Brucella* STA titer	IHA antibody titers against *Yersinia* antigens	
		O	ECA
B. abortus S19			
1	160	80	40
2	320	320	80
3	640	320	40
4	2,560	80	0
5	1,280	320	20
6	160	160	0
7	160	80	0
8	1,280	80	20
Y. enterocolitica O9			
1	640	640	1,280
2	320	640	1,280

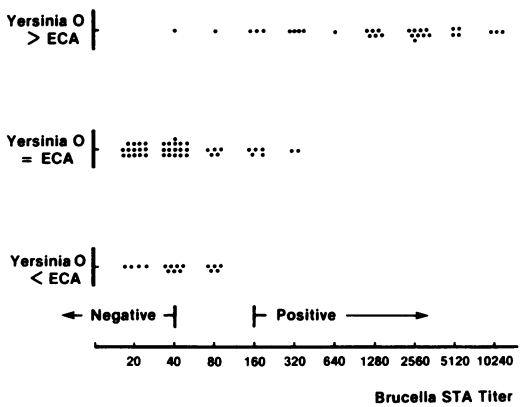

FIG. 1. *Relationship between relative anti-Yersinia O and anti-ECA IHA titers and the Brucella STA titer in 90 bovine sera. Each point represents a single sample.*

DISCUSSION

It is clear from the results obtained using laboratory animals that infection with *B. abortus* alone does not provoke the development of antibodies to ECA as measured by IHA. They will, however, develop as a result of exposure to *Y. enterocolitica* O9, and it is evident from the literature (6) that they will, in fact, develop in response to any enterobacterial infection. The presence of *Brucella* agglutinins in the absence of antibodies to ECA in an animal is therefore probably sufficient to exclude cross-reacting *Yersinia* as a cause of positive *Brucella* serology. However, because of the ubiquity of enterobacteria, it appears practically impossible to find cattle seronegative for ECA antibodies.

In the course of the investigations in calves it became apparent that a pattern existed insofar as *Brucella*-positive calves had a *Yersinia* O hemagglutinin titer which exceeded the ECA hemagglutinin titer, whereas in *Yersinia*-infected animals the reverse tended to apply. It should be pointed out that in the *Brucella*-infected experimental calves the *Yersinia* O titers tended to exceed the ECA titers by up to four dilutions; in the *Yersinia*-infected calves the difference was only an insignificant single dilution.

Analysis of the results from 90 bovine serum samples tended to confirm this tendency (Fig. 1). That is, *Brucella*-positive animals had relatively high titers to *Yersinia* O antigen, and in general, these titers tended to be considerably higher than the anti-ECA titer. However, seven animals which were unequivocally positive for *Brucella* by STA had anti-*Yersinia* O titers equal to the anti-ECA titers. Equality of titers must therefore be considered to be a nonconclu-

TABLE 3. *Full serological data on the two bovine sera, showing ambiguous results by IHA*

Animal no.	*Brucella* agglutination		*Yersinia* agglutination		Microplate agglutination		IHA *Yersinia*		
	STA	2-ME[a]	H	OH	*Brucella* O	*Yersinia* O	OH	O	ECA
65	40	40	20	40	40	40	40	40	10
294	80	0	20	80	20	20	80	320	40

[a] 2-ME, 2-Mercaptoethanol agglutination.

sive result. No *Brucella*-positive animals had anti-ECA titers which exceeded anti-*Yersinia* O titers.

In contrast, *Brucella*-negative animals had, with one exception, anti-ECA titers which equaled or exceeded their anti-*Yersinia* O titers. The single cow (no. 65) which had a relatively high anti-*Yersinia* O titer had an anti-*Brucella* titer of 1:40 which was totally resistant to 2-mercaptoethanol. The pattern of response of this sample in the microplate agglutination test was, in our experience, more compatible with a *Brucella* rather than a *Yersinia* infection (Mittal and Tizard, submitted for publication) (Table 3).

Within the group which had "suspicious" titers of antibodies to *Brucella* (1:80), five had a relatively high ECA titer, and this may imply either infection with a cross-reacting *Yersinia* or mixed infection. One animal (294) (Table 3) had a relatively high anti-*Yersinia* O titer. The *Brucella* agglutinins in this animal were susceptible to mercaptoethanol treatment. It also had a relatively high level of anti-*Yersinia* flagellar antibodies, which tends to suggest that it might in fact be a *Yersinia*-infected animal.

The presence of anti-ECA in the serum of an animal implies exposure to a type of enterobacteria. The presence of *Brucella* O agglutinins implies exposure to either *B. abortus* or a cross-reacting organism such as *Y. enterocolitica* O9. The presence of both can imply either *Y. enterocolitica* O9 infection or a mixed infection between *B. abortus* and any enterobacteria. This obviously places limits on the interpretation which can be made of these titers. Nevertheless, we believe that this technique is capable of providing information which is of assistance in interpreting suspicious reactions.

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