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Serological discrimination by indirect enzyme immunoassay between the antibody response to *Brucella* sp. and *Yersinia enterocolitica* O:9 in cattle and pigs

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Abstract

A rapid, inexpensive and rugged serological test that distinguishes cattle and swine infected with *Brucella* sp. or *Yersinia enterocolitica* O:9 is described. The test protocol, which is an indirect enzyme immunoassay uses a high concentration of divalent cation chelating agents to minimize binding of *Y. enterocolitica* O:9 antibody to rough lipopolysaccharide antigen derived from *B. abortus* RB51. No false positive reactions were observed when testing 100 Canadian cattle and swine without any evidence of brucellosis. The assay detected 91.6% of cattle (n = 155) and 93.5% (n = 31) of swine infected with *Brucella* sp. Sera from 58 cattle and 38 swine exposed to *Y. enterocolitica* O:9 were negative while only 20 sera from 121 'false positive' reactors of unspecified origin gave low level positive reactions, eliminating 84% of the false positive reactions.

Keywords: *Brucella* sp.; *Yersinia enterocolitica* O:9; Serology; IELISA; Differentiation

1. Introduction

A number of bacteria induce antibody responses that cause false positive reactions in tests for brucellosis, impeding accurate serological diagnosis. These bacteria include *Salmonella* group N (Corbel, 1979) and *Eschericia coli* O:157, *E. coli* O116 and *Pseudomonas maltophilia* (Corbel et al., 1984), however, the most notable cross reaction is between smooth lipopolysaccharide (SLPS) containing *Brucellae* and *Y. enterocolitica* O:9 (Ahvonen et al., 1969; Ahvonen and Sievers, 1969; Hurvell et al., 1971, 1979; Hurvell, 1973a,b,

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1975; Hurvell and Lindberg, 1973). This diagnostic problem resulted in a number of unsuccessful attempts to discriminate between the antibody responses to the two bacteria by indirect enzyme immunoassays (Carlsson et al., 1976; Hurvell et al., 1979) or conventional tests (agglutination and complement fixation tests) based on detection of antibody to the *O*-polysaccharide (OPS) epitopes of the smooth lipopolysaccharide molecule. These attempts were largely unsuccessful because of shared antigenic determinants in the OPS molecule, which is the basis for most serological tests (Caroff et al., 1984a,b; Bundle et al., 1984). Other protocols have been reported to be more successful. A periodate-oxidized and borohydride-reduced determinant of *Y. enterocolitica* O:9 was shown not to be shared with *B. abortus* (Lindberg et al., 1982) and an ELISA using *n*-lauroylsarcosine extracted antigen from *B. abortus* was also shown to distinguish antibody induced by *Y. enterocolitica* O:9 from that induced by *Brucella* sp. in various host species (Erdenebaatar et al., 2003). Cross reactivity between *Y. enterocolitica* O:9, *Y. enterocolitica* O:5 and *Y. enterocolitica* O:6 was used in an ELISA for differentiation between *Brucella* sp. and *Yersinia* sp. antibodies since *Y. enterocilitica* O:5 and *Y. enterocolitica* O:6 produced only a low level of cross reaction with *Brucella* antigens (Staak et al., 2000).

The anti-protein antibody response has also been investigated. This response is often delayed when compared to the SLPS response and was limited to animals, which developed an active brucellosis infection (experimentally infected, pregnant animals and sheep and goats from areas where brucellosis is still endemic) (Letesson et al., 1997). *Yersinia* sp. outer membrane protein (OMP) antigens distinguished in some cases in IELISA (Weynants et al., 1996), however, there was a high incidence of antibody to *Yersinia* OMP in brucellosis negative animals in Belgium and in New Zealand, restricting the use of these for epidemiological enquiries (Kittelberger et al., 1995; Weynants et al., 1996). Antibody to plasmid encoded OMPs of pathogenic *Yersinia* sp. was not detected in human brucellosis cases using immunoblots (Schoerner et al., 1990). OMPs from *B. melitensis* detected antibody in *B. meltensis* infected animals, but not in *Y. enterocolitica* O:9 infected sheep by Western blot (Corrente et al., 2004). Low level of antibody to a 18 kDa cytoplasmic protein (LPS free) was present in cattle vaccinated with *B. abortus* S19 or infected with *Y. enterocolitica* O:9, while higher responses were observed in animals infected with *B. abortus* S544 (Baldi et al., 1996). A 17 kDa protein from *B. melitensis* was found to be a common antigen with *Y. enterocolitica* O:9 while a 62 kDa protein (related to HSP-65 of *Mycobacteria*) was somewhat more selective (Spencer et al., 1994). The OMP31 antigen from *B. melitensis* was not found to be useful for serological diagnosis (Cassataro et al., 2004), however, differentiation of antibody response to *B. abortus* and *Y. enterocolitica* O:9 using H-agglutinins was possible (Mittal and Tizard, 1979, 1981).

Antigen binding cells in blood from *Brucella* sp. infected guinea pigs bind more *Brucella* SLPS than *Yersinia* SLPS (Karal'nik et al., 2002). A lymphocyte stimulation test using a polypeptide antigen (containing some LPS) was found to differentiate between *B. abortus* and *Y. enterocolitica* O:9 infection (Chukwu, 1985). Similarly, a skin test distinguished infection with *Brucella* from *Y. enterocolitica* O:9 (Saegerman et al., 1999; Godfroid et al., 2002). The interferon gamma test for brucellosis was found to be a good alternative to the delayed type hypersensitivity (skin) test using 'Brucellergen' (Weynants et al., 1995). The former test, using 'Brucellergen' as the antigen, was shown to distinguish cattle experimentally infected with *Y. enterocolitica* O:9 from *B. abortus* infection (Kittelberger et al., 1997) and swine, as reliably as the skin test (Riber and Jungersen, 2004).

While the structure of the OPS has been determined to be nearly identical for *B. abortus* and *Y. enterocolitica* O:9, some differences based on monoclonal antibody analysis have been described (Cloeckaert et al., 1993). The core oligosaccharide of the two species has been studied and found to be different. Thus, the *Brucella* core structure consists of 3-dideoxy-D-manno-2-octulosonic acid, mannose, glucose, glucosamine and quinovosamine (Moriyon et al., 1987; Velasco et al., 2000; Moriyon et al., 2004). The *Y. enterocolitica* O:9 core is L- or D-glycero-alpha-D-manno-heptapyranose and Kdo is 3-dideoxy-D-manno-oct-2-ulopyranosonic acid, with all hexoses being pyranoses (Muller-Loennies et al., 1999). Although *B. abortus* RB51 produces small amount of OPS (Cloeckaert et al., 2002) detectable by serological tests (Ciuchini et al., 2002; Nielsen et al., submitted for publication), it was decided in this study to ascertain if antibody to the core region of *B. abortus*

RB51 LPS could be used as an antigen for serological differentiation between *Y. enterocolitica* O:9 and *Brucella* sp.

2. Materials and methods

2.1. Serum samples

Serum samples ($n = 103$) were obtained from randomly selected Canadian cattle (Canada has been free from bovine brucellosis since 1985) and from 100 randomly selected Canadian swine (Canada has never had a case of swine brucellosis). These sera were considered as serologically negative controls. Sera from 58 cattle experimentally immunized with heat killed *Y. enterocolitica* O:9 were obtained as were 155 sera from cattle from which *B. abortus* was isolated or which originated from premises at which at least one animal was cultured positive for *B. abortus*. Sera from 38 pigs experimentally infected with *Y. enterocolitica* O:9 and 31 sera from pigs infected with *B. suis* were tested. The sera from *Brucella* infected cattle and pigs were obtained from other countries. Sera from 121 'false positive' brucellosis reactors all of which reacted in one or more of the standard tube agglutination test (SAT), the complement fixation test (CFT), the indirect enzyme immunoassay (IELISA) using SLPS from *B. abortus* or *Y. enterocolitica* O:9 or a competitive enzyme immunoassay (CELISA). All sera were stored at −20 °C.

2.2. Antigen extraction

The SLPS was extracted from *B. abortus* S1119.3 or *Y. enterocolitica* O:9 cells by the phenol:water procedure of Baker and Wilson (1965). Briefly, 5 g of dry *B. abortus* S1119.3 cells were treated with 190 ml 100% phenol and 170 ml distilled water at 66 °C for 20 min with constant agitation. After centrifugation (10,000 × *g* for 15 min at 4 °C), the phenol layer was removed, filtered to remove cellular debris and added to 500 ml cold methanol containing 1% (v/v) methanol saturated with sodium acetate. The resulting precipitate was removed by centrifugation as above and treated with 80 ml distilled water at 4 °C overnight with stirring. Crude SLPS in the supernatant solution was recovered by centrifugation at 10,000 × *g* for 15 min at 4 °C. The latter extraction was repeated with a further 80 ml of distilled water for 1 h and the two supernatant solutions were pooled after which 6.4 g trichoroacetic acid was added. The resulting precipitate was removed by centrifugation (10,000 × *g* for 10 min at 4 °C) and the supernatant solution containing relatively purified SLPS was dialyzed against water and freeze dried. The *Y. enterocolitica* O:9 cells were extracted similarly except the SLPS was recovered from the aqueous phase rather than the phenol phase.

The method described by Galanos et al. (1969) was used for preparation of RLPS. Twenty gram (wet weight) *B. abortus* RB51 cells were suspended in 250 ml petroleum ether:chloroform:phenol (anhydrous) in a ratio of 8:5:2 for 10 min with 10 bursts of homogenization of 10 s each. The soluble part was recovered by centrifugation at 5000 × *g* for 10 min at 4 °C. The pellet was re-extracted as above and the two soluble parts were pooled. The petroleum ether and the chloroform were allowed to evaporate and the phenol was dialyzed out using deionized water. A final concentration of 4% trichloroacetic acid was added and the resulting precipitate was removed by centrifugation (10,000 × *g* for 10 min at 4 °C). The supernatant solution was dialyzed and freeze dried.

2.3. Indirect enzyme immunoassay

Polystyrene 96 well plates (NUNC 69620) were passively coated with 100 ul *B. abortus* S1119.3 or *Y. enterocolitica* O:9 SLPS at 1 ug/ml or 100 ul of RLPS extracted from *B. abortus* RB51 at 5 ug/ml. All antigens were dissolved in 0.06 M carbonate buffer, pH 9.6 and incubated at 20 ± 2 °C for approximately 18 h after which the plates were frozen at −20 °C until used. Before use, the plates were thawed for about 45 min at ambient temperature. All remaining manipulations were done at ambient temperature (20 ± 2 °C). The plates were washed four times with 0.01 M phosphate buffer containing 0.15 M NaCl and 0.5% Tween 20, pH 7.2 (PBST). Serum samples (100 ul) diluted 1:50 in PBST containing 15 mM of each of EDTA and EGTA, pH 6.3 for the *B. abortus* S1119.3 and *Y. enterocolitica* O:9 SLPS assays were added to each well and incubated for 30 min. For the *B. abortus* RB51 RLPS assay, PBST containing 50 mM EDTA

and 50 mM EGTA, pH 6.3 was used to dilute serum samples 1:20. After four wash cycles with PBST, 100 ul of an appropriate amount of recombinant protein A/G conjugated with horseradish peroxidase (Nielsen et al., submitted for publication) diluted in PBST was added to each well for 1 h. Following a further four wash cycles with PBST, 100 ul of 1.0 mM H_2O_2 and 4 mM ABTS in 0.05 M citrate buffer, pH 4.5 were added and incubated with continuous shaking for 10 min after which an optical density reading was taken at 414 nm, except when the *B. abortus* RB51 antigen was used. In this case, the substrate and chromogen (75 ul) were 1.0 mM H_2O_2 and 4.2 mM TMB, shaken for 10 min followed by 75 ul 1.0 M H_2SO_4 stopping solution. Optical density readings were done at 450 nm. Data for the IELISA was recorded as percent positivity (%P) relative to a strongly positive control serum included for each species. Additional controls included a weakly positive serum and a negative serum as well as a control without serum.

2.4. Data analysis

Data was analyzed using MedCalc software (Schoonjans et al., 1995) for determination of sensitivity and specificity values. For the *B. abortus* and *Y. enterocolitica* O:9 SLPS IELISA tests, a cutoff of 10%P (10% of the optical density of a strong positive serum reaction) was selected based on previous determinations (Nielsen et al., submitted for publication) while the cutoff for the IELISA using *B. abortus* RB51 RLPS was set at 20%P. Individual results for each serum sample for individual antigens were plotted in an interactive dot analysis.

3. Results

Fig. 1 depicts the results obtained with cattle sera in the IELISA. Fig. 1A represents the %P values obtained with *B. abortus* and *Y. enterocolitica* O:9 positive and negative cattle using and *B. abortus* S1119.3 SLPS as the antigen. A cutoff of 10%P resulted in 100% positive reactions for both populations of animals. No sera from the negative group gave results above this cutoff. Please note that a considerable range of %P was included to avoid using solely sera with very high antibody levels for this study. Fig. 1B represents the same serum samples as above but using *Y. enterocolitica* O:9 SLPS as the antigen. A cutoff of 10%P resulted in 99% of both the *Brucella* and the *Yersinia* exposed groups giving positive reactions and specificity values of 100%. In Fig. 1C, the same sera were tested against *B. abortus* RB51 RLPS antigen. Using a cutoff value of 20%P, 142 of the 155 (91.6%) *B. abortus* positive sera tested gave a positive result while none of the 58 sera from *Y. enterocolitica* O:9 positive animals gave results above this cutoff (specificity is 100%). None of the 100 Canadian sera reacted above the cutoff (specificity is 100%).

Results obtained with pig sera are presented in Fig. 1D–F. The sensitivity with sera from pigs exposed to *B. suis* was 100% while antibody in 89.5% (specificity 10.5%) of sera from *Y. enterocolitica* O:9 exposed pigs was detected with SLPS antigens from *B. abortus* S1119.3. No Canadian pig sera reacted above 10%P (specificity 100%) (Fig. 1D). Using *Y. enterocolitica* O:9 SLPS antigen, all sera from *B. suis* and *Y. enterocolitica* O:9 exposed pigs reacted giving sensitivity and specificity values of 100 and 0% for the two groups. No sera from Canadian pigs reacted above the cutoff value (Fig. 1E). As was the case with bovine sera, none of the 38 sera from *Y. entercolitica* O:9 exposed pigs reacted with *B. abortus* RB51 RLPS while 29 of 31 (93.5%) *B. suis* infected animals gave reactions above the 20%P cutoff (Fig. 1F).

The results of testing 121 ‘false positive’ bovine brucellosis reactors sera are presented in Fig. 2. These sera all reacted in one or more of the commonly used diagnostic serological tests. Of the 121 sera, 20 were completely negative in the CFT and 23 were completely negative in the SAT (Fig. 2A). In the IELISA, using *B. abortus* SLPS as the antigen, 17 sera were negative and 12 sera gave negative reactions in IELISA using *Y. enterocolitica* O:9 SLPS. Using *B. abortus* RLPS antigen, 101 of the sera were negative. These data are presented in Fig. 2B. The 20 sera that gave positive reactions in the *B. abortus* RLPS IELISA gave a range of results in the other tests, even a few negative reactions. Of these sera, only one did not appear to contain antibody to *Y. enterocolitica* O:9 SLPS. These data are presented in Table 1.

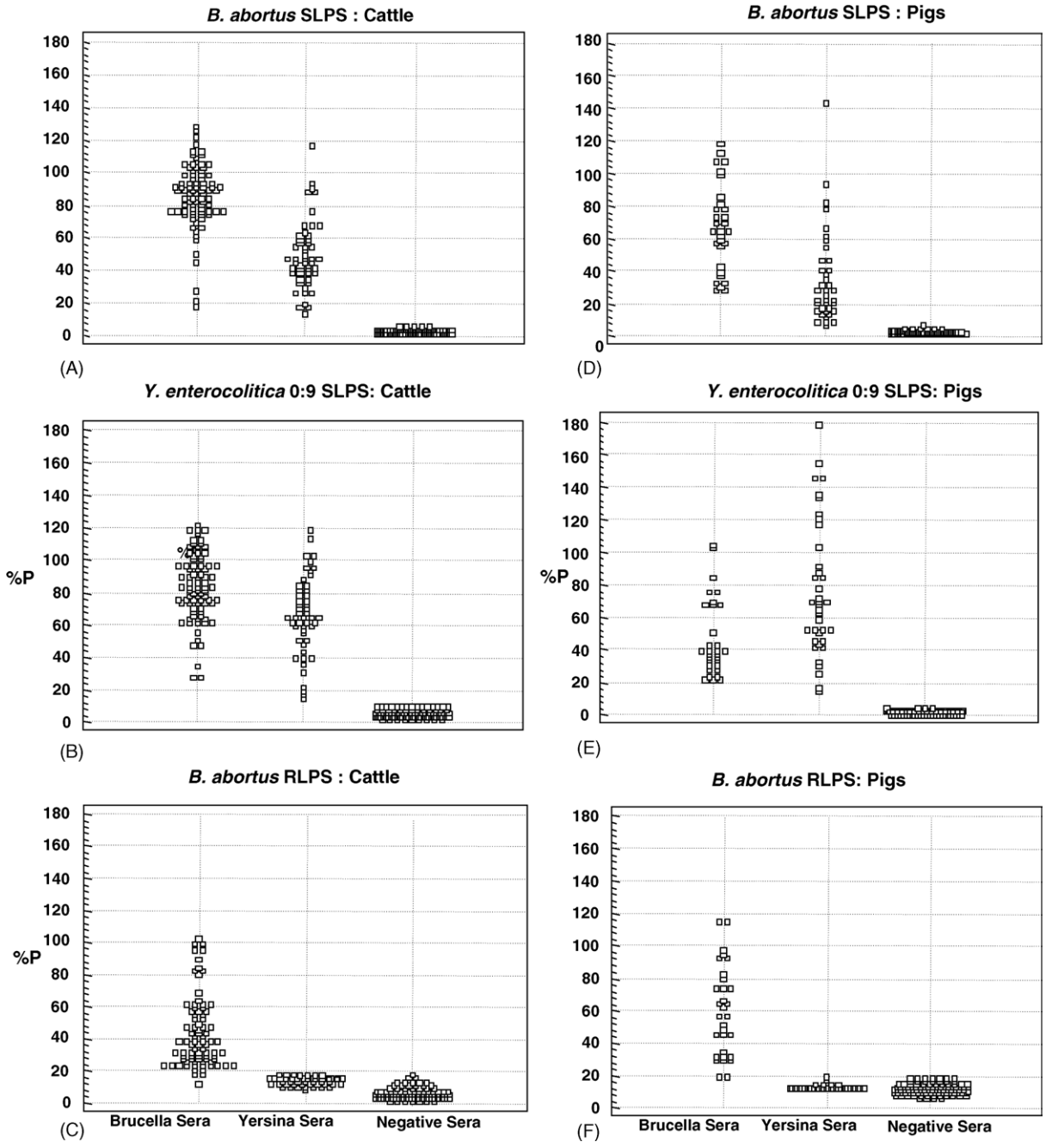

Fig. 1. Interactive dot analysis of serum samples tested by indirect enzyme immunoassay for antibody to *B. abortus* SLPS, *Y. enterocolitica* O:9 SLPS and *B. abortus* RB51 RLPS antigens. Sera were collected from cattle and pigs exposed to *B. abortus*, *Y. enterocolitica* O:9 or unexposed. The cutoff values for the IELISA for antibody to *B. abortus* and *Y. enterocolitica* O:9 SLPS was 10%P and 20%P for *B. abortus* RB51 RLPS. Panel A depicts the results obtained with the three groups of bovine sera tested against *B. abortus* SLPS; panel B depicts the same sera tested with *Y. enterocolitica* SLPS and panel C is the data obtained when the sera were tested with *B. abortus* RB51 RLPS. Panel D represents swine sera from animals exposed to *Brucella* sp., *Y. enterocolitica* O:9 or unexposed and tested with *B. abortus* SLPS. Panel E is the data obtained when the three groups of sera were tested with *Y. enterocolitica* O:9 SLPS and panel F when the sera were tested with *B. abortus* RB51 RLPS.

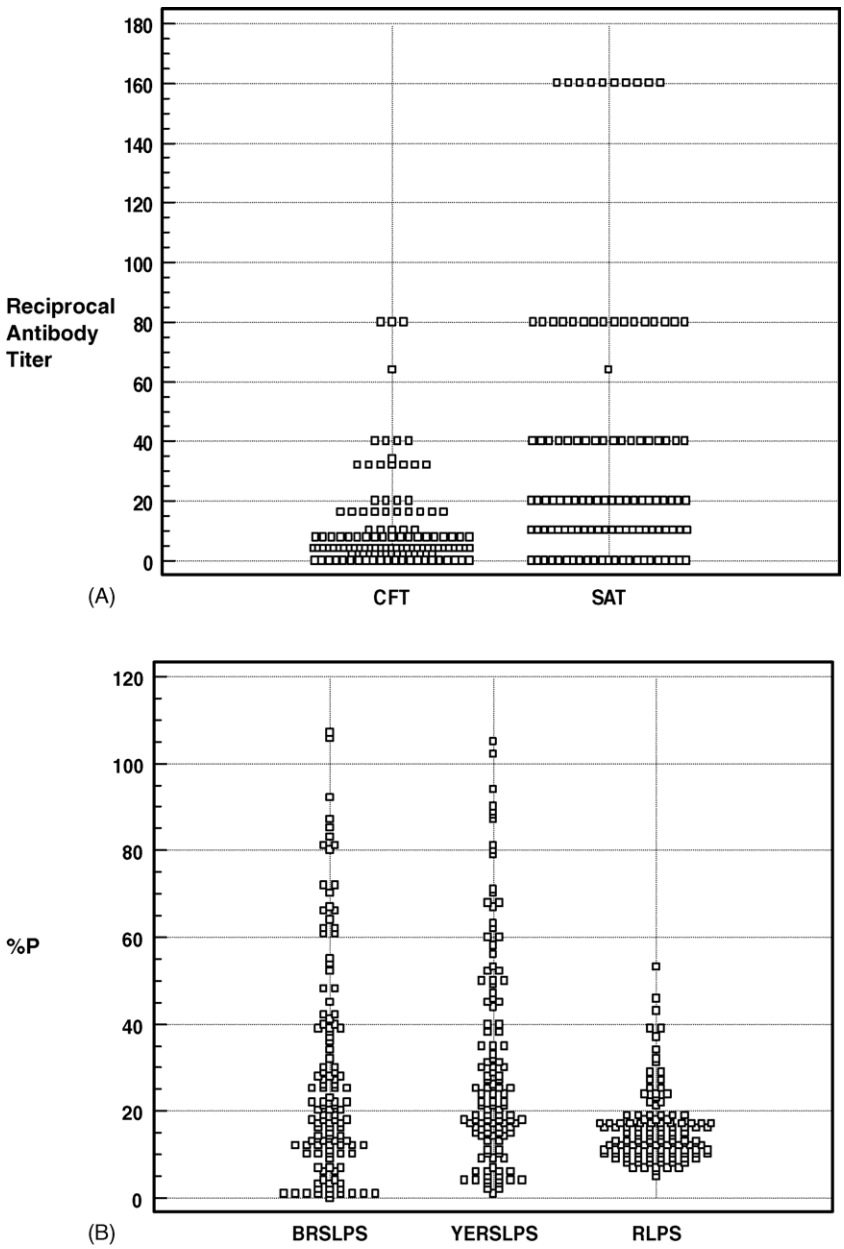

Fig. 2. Interactive dot analysis of 121 'false positive' brucellosis reactor sera in serological tests. (A) Represents the data using the complement fixation test (CFT) and the standard tube agglutination test (SAT). Please note that three and five sera gave titers above the 1:160 serum dilution in the CFT and SAT, respectively. These points were excluded to avoid compression of the data. (B) Includes the data obtained in the indirect enzyme immunoassay using *B. abortus* SLPS (BRSLPS) and *Y. enterocolitica* O:9 SLPS (YERSLPS) and *B. abortus* RLPS (RLPS) as the antigens. The cutoff for the SLPS ELISAs is 10%P while the cutoff for the RLPS ELISA is 20%P.

Table 1
Reactivity of 20 'false positive' sera that were positive in the *B. abortus* RLPS IELISA in various serological tests

Serum number	CFT	SAT	CELISA	IELISA SLPS	IELISA YE09	IELISA RLPS
1	20	160	35	27	38	39
5	80	80	99	107	102	43
8	2	80	19	4	22	29
15	40	160	73	72	68	24
21	20	640	99	92	90	25
27	200	320	99	67	87	46
35	4	80	51	13	10	31
37	40	80	85	72	71	24
39	–	80	7	1	10	32
42	2	10	35	22	28	22
43	8	–	25	28	17	35
55	8	10	17	30	18	39
66	32	40	78	106	67	27
71	8	10	21	19	16	21
72	4	10	15	17	13	23
76	–	–	21	37	35	34
82	–	10	31	12	15	37
88	2	–	21	14	16	29
93	8	10	22	18	19	25
99	16	20	57	62	49	27
119	–	40	6	10	3	53

CFT refers to the complement fixation test (reciprocal of dilution giving a 2+ reaction or higher), SAT is the standard tube agglutination test (reciprocal of dilution giving at least 50% agglutination), CELISA is the competitive ELISA (% inhibition relative to an uninhibited control, 30% or higher is positive), IELISA SLPS used *B. abortus* SLPS antigen (% positivity relative to a positive control, 10% or higher is positive), IELISA YE O9 used *Y. enterocolitica* O:9 SLPS antigen (% positivity relative to a positive control, 10% or higher is positive), IELISA RLPS used *B. abortus* RLPS antigen (% positivity relative to a positive control, 20% or higher is positive), (–) indicates a negative result.

4. Discussion

The *Brucella* SLPS IELISA used in this study was unable to differentiate brucellosis from *Y. enterocolitica* O:9 infection and thus confirmed previous reports (Garin et al., 1997; Saegerman et al., 1999). In fact, both the *Brucella* and *Yersinia* exposed animals were consistently detected by this assay. In addition, a similar IELISA, using SLPS from *Y. enterocolitica* O:9 was generally capable of detecting the same animals as the *Brucella* SLPS IELISA. Therefore, for brucellosis control and eradication programs, other tests are required. While it is possible to differentiate *Y. enterocolitica* O:9 infection from brucellosis by using lymphocyte proliferation tests, these protocols are time consuming, expensive and time sensitive. Therefore, a rapid, inexpensive and rugged test, normally associated with serological procedures, would be more suitable to large-scale diagnosis. In earlier investigations, it was noticed that sera from *Brucella* sp. infected animals frequently reacted with a core preparation from the RLPS of *B. abortus* RB51 and interestingly, sera from animals exposed to rough *Brucella* sp. often reacted with SLPS from *B. abortus* S1119.3. These observations indicated that although *B. abortus* RB51 may contain some OPS in its RLPS, antibody from animals infected with smooth *Brucella* sp. was capable of recognizing the core region of RLPS. In addition, antibody to rough *Brucella* sp. reacted with SLPS, presumably through its core region as well. While the OPS of smooth *Brucella* sp. and *Y. enterocolitica* O:9 are nearly identical, information available on the structure of the core regions of the two bacterial species indicated considerable differences that could be useful for antibody response differentiation. From the data presented, the measurement of antibody to *B. abortus* RB51 core in the presence of a high concentration of divalent cation chelating agents eliminates reactivity by antibody to *Y. enterocolitica* O:9. It is clear from the data that this procedure can be used to eliminate reactions in bovine and porcine sera from animals experimentally exposed to *Y. enterocolitica* O:9 in over 90% of the cases with little or no loss of

diagnostic capability for detection of antibody to *Brucella* sp. The 13 of 155 cattle sera and the 2 of 31 porcine sera that gave negative results with the *B. abortus* RB51 RLPS were obtained from animals from which *Brucella* could not be isolated, however, the organism was isolated from other animals on the premise. It is very likely that those sera came from animals in the very early stages of infection where there is likely only a relatively low level of antibody to the core region.

Of 121 sera collected from cattle with 'false positive' brucellosis tests, 20 resulted in positive reactions in the RLPS IELISA. The range of results in this test, from 21 to 53% positivity did not correlate well with results from the other serological tests (Table 1). Only the SLPS and YE09 ELISA results seemed to correlate reasonably well. The reason for this low sporadic reactivity with RLPS observed in some sera is unknown. As the sera tested were considered not to be from animals with brucellosis, the reactivity must be due to antibody resulting from stimulation of the antibody response by antigens with similar epitopes or alternately some serum protein idiosyncracy.

Table 2 summarizes the most likely specificity of the antibodies against LPS produced during infection or vaccination with *Brucella* sp. and *Y. enterocolitica* O:9. In the early stages of infection, there is likely only a relatively low level of antibody to the core region while there is substantial antibody to the OPS. In later stages of infection, diagnostically significant levels of anti-core antibody are present and in nearly all cases, very high and sustained levels of antibody to OPS. While only a handful of sera from *B. abortus* S19 vaccinated animals have been tested, antibody to the core regions was not in evidence, probably because of the relatively short time of exposure. This phenomenon should be further investigated in order to evaluate if this newly developed test is able to differentiate *Brucella* infected animal from animals vaccinated with *B. abortus* S19. Vaccination with *B. abortus* RB51 results in relatively high but transient levels of antibody to the core regions and low levels of anti-OPS antibody (unpublished data). Experimental exposure to *Y. enterocolitica* O:9 results in significant antibody levels to OPS but no measurable antibody to *B. abortus* RB51 core.

Table 2
Proposed antibody specificity after exposure to *Brucella* sp., pathogenic or vaccine strains and to *Y. enterocolitica* O:9

		Antibody against	
		O-Polysaccharide	Core-lipid A
Brucella abortus			
Infection	Early	++	(+)
Infection	Late	++++	++
Vaccination	S19	+++	(+)
Vaccination	RB51	(−)	++
Yersinia enterocolitica O:9			
Infection		++++	–

'+' Indicates the magnitude of the antibody response; '(+)' indicates minor if any measurable antibody production; '−' indicates not measurable antibody.

It is realized that some important points require investigation, including assessment of sera from natural *Y. enterocolitica* O:9 infection and natural infections with different *Brucella* species and biovars, investigation of reactivity of other cross reacting microorganisms and assessment of the protocol with sera from other species. Such samples, however, were not available but would be welcome for further studies.

In summary, a test for rapid, inexpensive and accurate diagnosis of brucellosis in cattle and pigs is described. This test has the capacity to exclude animals exposed to *Y. enterocolitica* O:9 in more than 90% of the sera tested and 84% of 'false positive' brucellosis reactors of unknown origin. Therefore, this test can be used either as a screening or as a confirmatory test as part of a testing strategy. Indeed, in cattle, the proportion of serological cross reactions in the brucellosis serological tests induced by *Y. enterocolitica* O:9 is increasing in the later stages of control or eradication programs. Therefore, other tests or combinations of tests have been used in addition to proficient epidemiological enquiries (Godfroid et al., 2002). This RLPS IELISA should be further validated under these epidemiological circumstances. In pigs, none of the conventional serological tests used for the diagnosis of porcine brucellosis are reliable for diagnosis in individual pigs due to the common occurrence of *Y. enterocolitica* O:9 in some areas (OIE Manual, 2004). Therefore, the RLPS IELISA should be further validated as a screening test for this species as well. The same test would be useful for a wide range of species, particularly wildlife, due to the

use of a protein A/G enzyme conjugate as a detection reagent (Nielsen et al., submitted for publication).

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