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Towards single screening tests for brucellosis

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Summary

This paper describes an indirect enzyme-linked immunosorbent assay (I-ELISA) and a fluorescence polarisation assay (FPA), each capable of detecting antibody in several species of hosts to smooth and rough members of the genus *Brucella*. The I-ELISA uses a mixture of smooth lipopolysaccharide (SLPS) and rough lipopolysaccharide (RLPS) as the antigen, and a recombinant protein A/G conjugated with horseradish peroxidase as the detection reagent. When using individually determined cutoff values, the SLPS/RLPS combined-antigen I-ELISA detected antibody in slightly more animals exposed to SLPS or to RLPS than did I-ELISA procedures using each individual antigen separately. Similarly, the assay using combined antigens detected antibody in slightly fewer animals not exposed to *Brucella* sp. When a universal cutoff of 10% positivity was used (relative to strongly positive control sera of each species), the overall performance index (percentage sensitivity plus percentage specificity) value decreased by 1.0 (from 199.4 to 198.4). In the FPA, it was not possible to use a universal cutoff without significant loss of performance. The overall sensitivity value for the FPA using the combined FPA antigen was 1.0% lower than using the O-polysaccharide (OPS) from SLPS and 9.1% higher than using the core antigen (CORE) from RLPS. When the combined antigen was used, the FPA specificity was slightly higher (1.2%) than from only the OPS, and considerably higher (12.6%) than the CORE. Overall, both the I-ELISA and the FPA with combined antigens were suitable as screening tests for all species of *Brucella* in the animal species tested.

Keywords

Brucellosis – Diagnosis – Fluorescence polarisation assay – Indirect enzyme-linked immunosorbent assay – Serology – Universal assay.

Introduction

The species of the genus *Brucella* have traditionally been thought to be relatively restricted in their host specificity. While this is true to some extent, a substantial number of cross-species infections occur. *Brucella canis*, a recent addition to the genus (6), was initially associated with infection in dogs, but there have been reports of infections in both humans (22, 25) and cattle (13), although humans and cattle are thought to be relatively resistant to *B. canis* infection (5). Similarly, most of the *Brucella* sp. containing smooth lipopolysaccharide (SLPS) can infect multiple species. An example is *B. suis*, which has been isolated from humans (14), cattle (7, 8), dogs (2, 10, 27), horses (23) and sheep (20) in addition to pigs.

In some cases, hosts are infected with species containing both smooth and rough LPS. The classical example is sheep infected with *B. ovis* (3) or *B. melitensis* (1), or perhaps both. This led to questions by Godfroid and Kasbohrer (9) regarding the detection and management of false negative reactions. Uncertainty about the infecting species could easily lead to misdiagnosis when using serological tests. Most tests are designed to detect either smooth or rough LPS, not only in domestic animals but also in humans and wildlife. Therefore, a universal screening test may be very useful for the initial determination of the presence of antibody to the genus. If positive, such a test could then be followed up with one or a panel of more specific tests to determine the infecting bacterial species.

Two robust serological tests, the indirect enzyme-linked immunosorbent assay (I-ELISA) and the fluorescence polarisation assay (FPA) were investigated to ascertain if they could be adapted to a single screening test for several hosts and both smooth and rough bacterial species. Rough and smooth LPS were mixed for use as the antigen, and recombinant protein A/G conjugated with horseradish peroxidase was used as a universal detection reagent in the I-ELISA. For the FPA, O-polysaccharide (OPS) from SLPS and CORE region of rough lipopolysaccharide (RLPS) were labelled with a fluorochrome, mixed and tested in the FPA. Both tests were applied to sera from a number of species that had been infected or exposed to various *Brucella* sp., and to sera from animals of the same species that had not been exposed to *Brucella* sp.

Materials and methods

Serum samples

Samples from dogs, sheep and cattle exposed to *B. canis*, *B. ovis* and *B. abortus* RB51 (rough strains), respectively, and samples from sheep and goats exposed to *B. melitensis*, pigs exposed to *B. suis* and cattle exposed to *B. abortus*, as well as bison and deer species exposed to either *B. abortus* or *B. suis* biovar 4 (smooth strains) were tested. In all cases, except animals exposed to *B. abortus* RB51, sera were derived from naturally infected animals from infected farms (*Brucella* sp. was isolated from the animal itself or from at least one animal on the premises). Cattle exposed to *B. abortus* RB51 were immunised with killed bacteria. Sera from non-exposed members of the same species, which were used as negative controls, were of Canadian origin and from premises with no indication of brucellosis. (The number of sera from each species is indicated in Tables Ia, Ib, Ic, Id.)

Indirect ELISA

Polystyrene 96-well plates were passively coated with 100 µl *B. abortus* smooth LPS extracted from S1119.3 at 1 µg/ml, 100 µl of rough LPS extracted from *B. abortus* RB51 at 5 µg/ml or 100 µl of an equal mixture of the two reagents. All antigens were dissolved in 0.06M 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 they were used. Before use, the plates were thawed for about 45 min at ambient temperature (20 ± 2°C). All remaining manipulations were done at ambient temperature. The plates were washed four times with 0.01M phosphate-buffered saline (PBS) containing 0.15M NaCl and 0.5% Tween 20, pH 7.2 (PBST). Serum samples (100 µl) diluted 1:50 in PBST containing 15 mM of each of ethylenediamine tetra-acetic acid and ethylene glycol tetra-acetic acid, pH 6.3 were added to each well and incubated for 30 min. After four wash cycles with PBST, 100 µl of an appropriate amount of recombinant protein A/G conjugated with horseradish peroxidase diluted in PBST was added to each well for 1 h. Following a further four wash cycles with PBST, 100 µl of 1.0 mM H_2O_2 and 4mM 2,2'-azino-bis (3-ethyl benzthiazoline-6-sulfonic acid) in 0.05M 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.

Data for the I-ELISA was recorded as percentage 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.

Protein AG construction

Staphylococcus aureus (ATCC #12598) and the group G *Streptococcus* sp. (ATCC #12394) were purchased from ATCC, and chromosomal deoxyribonucleic acid (DNA) from each bacterium was obtained from overnight cultures.

Table I

Number of animals tested for each host species, exposed to Brucella sp. (Table Ia) and unexposed (Table Ib) with the numbers resulting in positive (Ia) or negative (Ib) reactions in the indirect enzyme-linked immunosorbent assays I-ELISA using smooth lipopolysaccharide (SLPS), rough lipopolysaccharide (RLPS), or SLPS and RLPS antigens combined with a protein A/G enzyme conjugate.

The same data is presented for the fluorescence polarisation assay (Tables Ic and Id) using O-polysaccharide (OPS), CORE or OPS/CORE antigens. The percentage sensitivity (Ia and Ic) and percentage specificity (Ib and Id) for the total number of sera tested from all host species were calculated.

***a*) Sensitivity of the I-ELISA**

Animals indicated according to exposure agent Species	Number exposed	Number of positive responders using		
		SLPS	RLPS	R/SLPS
Cattle SLPS	71	70	61	71
Swine SLPS	74	73	51	73
Bison SLPS	102	102	86	102
Goats SLPS	68	68	68	68
Deer SLPS	64	64	64	64
Sheep SLPS	70	62	42	70
Cattle RLPS	60	42	60	60
Dogs RLPS	21	21	21	21
Sheep RLPS	87	87	87	87
Combined total	617	589	540	616
Percentage sensitivity		95.5	87.7	99.9

RLPS: rough lipopolysaccharide
SLPS: smooth lipopolysaccharide

***b*) Specificity of the I-ELISA**

Animals indicated according to exposure agent Species	Number not exposed	Number of negative responders using		
		SLPS	RLPS	R/SLPS
Cattle SLPS	79	79	78	79
Swine SLPS	72	72	69	72
Bison SLPS	163	162	144	162
Goats SLPS	80	80	72	79
Deer SLPS	78	78	74	77
Sheep SLPS	60	60	58	60
Cattle RLPS	79	71	79	79
Dogs RLPS	80	75	78	78
Sheep RLPS	58	58	58	58
Combined total	749	735	710	744
Percentage specificity		98.1	94.8	99.5

RLPS: rough lipopolysaccharide
SLPS: smooth lipopolysaccharide

***c*) Sensitivity of the FPA**

Animals indicated according to exposure agent Species	Number exposed	Number of positive responders using		
		OPS	CORE	OPS/CORE
Cattle SLPS	71	71	68	71
Swine SLPS	74	73	26	68
Bison SLPS	102	101	86	100
Goats SLPS	68	67	65	68
Deer SLPS	64	63	38	59
Sheep SLPS	70	66	33	65
Cattle RLPS	60	60	60	57
Dogs RLPS	N/A	N/A	N/A	N/A
Sheep RLPS	93	82	87	90
Total combined	602	583	463	578
Percentage sensitivity		97.0	76.9	96.0

N/A: not applicable
OPS: O-polysaccharide
RLPS: rough lipopolysaccharide
SLPS: smooth lipopolysaccharide

***d*) Specificity of the FPA**

Animals indicated according to exposure agent Species	Number not exposed	Number of negative responders using		
		OPS	CORE	OPS/CORE
Cattle SLPS	79	79	78	79
Swine SLPS	72	71	28	72
Bison SLPS	163	158	150	161
Goats SLPS	80	80	56	80
Deer SLPS	78	78	76	78
Sheep SLPS	60	59	60	60
Cattle RLPS	79	79	78	79
Dogs RLPS	NA	NA	NA	NA
Sheep RLPS	58	57	56	57
Total combined	669	661	582	666
Percentage specificity		98.8	87.0	99.6

The cell pellets were suspended in 200 µl of PBS (pH 7.2) containing 0.25 µg of lysozyme and incubated at 37°C for 1 h. Then, 50 µl (20 mg/ml) of proteinase K was added and incubated for another hour. Lysis buffer (600 µl of 0.73% cetyltrimethylammonium bromide, 0.57M of NaCl in PBS, pH7.2) was added to the cell lysate, and DNA was extracted with phenol/chloroform/isoamyl alcohol in the ratio of 25:24:1, washed with 70% ethanol, dissolved in water and kept at –20°C.

Fragments of DNA corresponding to the protein A (fragments A and B, containing two IgG binding repeats [26]) and to the protein G fragment (corresponding two IgG binding regions [12]) were generated by polymerase chain reaction (PCR) using an expanded high-fidelity PCR system. The PCR product for protein A or G was amplified by using 200 µM each of the deoxynucleotides, 25 pmol of protein A forward primer 5'– ACAGGATCCGCTGATAACA ATTTCAACAAAGAA-3' and reverse primer 5'- ACAGTCC ATGGGTTTTGGTGCTTGAGCATCATTTAAG-3'; or 25 pmol of protein G forward primer 5'- ACACCCATGGACA CTTACAAATTAATCCTTAATGGT-3' and reverse primer ACAGCGGCCGCATTCAGTTAC CGTAAAGGTCTTAGT-5' – in total 100 µl.

The amplification was achieved using: 94°C for 2 min; 35 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C; followed by a final extension at 72°C for 10 min. The PCR-generated protein A and G gene products were digested separately with *Nco*I and ligated with T_4 DNA ligase. The PCR product for protein A/G was generated by using protein A forward primer and protein G reverse primer by the same procedure as above. The purified DNA fragment of protein A/G was digested with *Nde*I and *Not*I and ligated into pET30a, which was also digested with *Nde*I and *Not*I. The ligation mixture was transformed into $DH_{5\alpha}$ according to the manufacturer's recommendations. The inserts were selected with colony PCR (95°C for 5 min; 35 cycles of 1 min at 94°C, 45 s at 56°C and 30 s at 72°C; followed by 72°C for 10 min by using T7 promoter and terminator primers) and analysed by 1% agarose gel electrophoresis. The plasmid DNA was extracted by using the QIAprep Spin Miniprep Kit (QIAGEN Inc., Missassauga, Ontario, Canada) and sequenced (Canadian Molecular Research Services Inc., Ottawa, Ontario, Canada).

Escherichia coli BL21 (DE3) cells containing the recombinant protein A/G plasmids were grown in LB broth containing 30 µg/ml kanamycin A. The expression of protein A/G was induced by adding isopropyl-beta-D-thiogalacto-pyranoside to a final concentration of 0.5 M, followed by incubation for 3 h at 37°C. Protein A/G was purified by means of a nickel-nitrilotriacetic acid Superflow Column (Qiagen Inc., Missassauga, Ontario, Canada) and labelled with horseradish peroxidase (11).

Fluorescence polarisation assay

Sera were diluted 1:25 in 0.01 M phosphate buffer, pH 6.5, in a 10 mm × 75 mm glass tube. Indigenous fluorescence was measured in a Sentry 1000 FPA analyser (Diachemix LLC, Milwaukee, Wisconsin, USA). After addition of 10 µl of fluorescein isothiocyanate-labelled OPS from smooth LPS, CORE region of rough LPS (CORE) or 10 µl of an equal mixture, and incubation for a minimum of 2 min, a final polarisation measurement was done. The analyser automatically subtracted the background fluorescence, providing a result in millipolarisation units (mP). Strongly positive, weakly positive and negative control sera were included for each host species, as well as a control without serum.

Data analysis

Data was analysed by receiver operator characteristic (ROC) analysis using MedCalc software (24). For each antigen in the two tests and for each species, the cutoff that resulted in the maximum sum of the sensitivity and specificity values was calculated. Similarly, for the I-ELISA and the FPA, the sensitivity and specificity values for universal cutoffs of 10%P and 90 mP were calculated. The latter calculation did not produce meaningful data. The percentages of sensitivity and specificity were added, giving a performance index (PI) for easier overall test evaluation. A Youden's plot was also prepared.

Performance indices were calculated as follows: the numbers of sera from each species from positive (exposed) animals or negative (non-exposed) animals in each test with each antigen were added, and a percentage of the total number of sera tested was calculated for point sensitivity and specificity values. These values were then added to provide PI for each antigen or antigen combination in each test.

Results

Validated I-ELISA and FPA protocols are already in use as prescribed tests for cattle. These tests were developed for detection of antibody to SLPS. The format described in this communication allows detection of antibody to SLPS and RLPS simultaneously in a large number of different species. This provides a global estimate of antibody, and should eliminate misdiagnosis when animals are infected with an unexpected species of *Brucella*.

In general, the I-ELISA performed efficiently with high levels of sensitivity and specificity when the optimum cutoff between positive and negative reactions was used (as determined for each antigen and each species by means of

ROC analysis). These data are presented in Table IIa. Of the sera from 71 cattle exposed to SLPS, 70 were identified as positive by using the SLPS antigen, and 61 sera were found to be positive using the RLPS antigen. All 71 sera gave positive results with the combined antigen. The results were similar for the other species exposed to smooth *Brucella* sp., with a substantial number of positive reactions, resulting in RLPS and the combined antigen giving results very similar to those of the SLPS antigen. Animals exposed to rough *Brucella* sp. gave a high reaction rate with RLPS antigen and also had a high rate of reaction with SLPS. Sera from dogs and sheep gave positive reactions with both antigens and with the combined antigens. Sera from cattle exposed to *B. abortus* RB51 all reacted positively with RLPS and the combined antigens. Of the 60 sera from this group, 42 reacted with SLPS antigen. The sera from non-exposed cattle tested with RLPS or the combined antigens gave no positive reactions; however, a substantial number of false positive reactions (10.1%) were observed in the SLPS I-ELISA. The reason for this is not understood, but may be partly a result of a cutoff of 10%P being used for the RLPS exposed cattle tests (Index 19, Table IIa) and a cutoff of 14%P being used for the SLPS assay (Index 1, Table IIa).

Using a universal cutoff of 10%P resulted in slight reductions in the PIs (gain/loss) of bison (–2.5), deer (–1.6) and dogs (–3.6). The overall sensitivity and specificity values shown in Tables Ia and Ib were obtained by adding the results from all species of positive or negative animals for each of the three

Table IIa
Results of the indirect enzyme-linked immunosorbent assay using sera from different host species, both exposed and unexposed to *Brucella* sp., tested with smooth lipopolysaccharide (SLPS), rough lipopolysaccharide (RLPS), or SLPS and RLPS combined with an enzyme-conjugated protein A/G detection reagent

Species	Index	Antigen	Cutoff	% sensitivity	% specificity	PI	PI (10%P)	Gain/loss
Cattle SLPS	1	SLPS	14	98.6	100.0	198.6	191.1	
	2	RLPS	9	85.9	98.7	184.6	184.6	
	3	S/RLPS	6	100.0	100.0	200.0	200.0	0
Swine SLPS	4	SLPS	3	98.6	100.0	198.6	198.6	
	5	RLPS	4	68.9	95.8	164.7	143.2	
	6	R/SLPS	4	98.6	100.0	198.6	198.6	0
Bison SLPS	7	SLPS	14	100.0	99.4	199.4	193.3	
	8	RLPS	10	84.3	88.3	172.6	172.6	
	9	R/SLPS	15	100.0	99.4	199.4	196.9	–2.5
Goats SLPS	10	SLPS	11	100.0	100.0	200.0	198.7	
	11	RLPS	4	100.0	90.0	190.0	172.0	
	12	R/SLPS	9	100.0	98.7	198.7	198.7	0
Deer SLPS	13	SLPS	4	100.0	100.0	200.0	200.0	
	14	RLPS	4	100.0	94.9	194.9	179.9	
	15	R/SLPS	8	100.0	98.7	198.7	197.1	–1.6
Sheep SLPS	16	SLPS	5	88.6	100.0	188.6	185.7	
	17	RLPS	3	62.9	96.7	159.6	148.6	
	18	R/SLPS	10	100.0	100.0	200.0	200.0	0
Cattle RLPS	19	SLPS	10	71.7	89.9	161.6	161.6	
	20	RLPS	13	100.0	100.0	200.0	198.7	
	21	R/SLPS	6	100.0	100.0	200.0	200.0	0
Dogs RLPS	22	SLPS	6	100.0	93.7	193.7	184.4	
	23	RLPS	11	100.0	97.5	197.5	196.2	
	24	R/SLPS	6	100.0	97.5	197.5	193.9	–3.6
Sheep RLPS	25	SLPS	5	100.0	100.0	200.0	197.7	
	26	RLPS	4	100.0	100.0	200.0	197.7	
	27	R/SLPS	10	100.0	100.0	200.0	200.0	0

The left column refers to the species tested and the type of *Brucella* sp. to which the host was exposed (SLPS containing species, RLPS Brucellae)
The cutoff refers to the percentage positivity giving the maximum sum of the percentage sensitivity and the percentage specificity values
The performance index (PI) is the sum of the percentage sensitivity and the percentage specificity
PI (10%P) refers to the value obtained using a universal cutoff for all indirect enzyme-linked immunosorbent assays of 10%P
Gain/loss refers to the difference between the PI derived from optimum cutoff values and those derived from the PI (10%P)

antigens in the I-ELISA. The sensitivity and specificity values, combined into PIs (Table III), indicated a gain in performance from using the combined antigens with the individually calculated cutoff values and with the universal cutoff value of 10%P. There was a slight decrease in PIs from 199.4 to 198.4 (–1.0) for the combined antigen. The PI using RLPS is lower because a number of animals exposed to smooth *Brucella* sp. did not produce a positive antibody response to this antigen, and similarly, the PI for SLPS was lower than the PI for the combined antigens.

In the FPA, the assay using the CORE antigen gave a PI of 74 for swine. A review of the ROC analysis showed that there were more negative reactors in the positive population than positive reactors, and more positive than negative reactors in the negative population, based on the cutoff value of 88. Other low PI values obtained by using CORE antigen were with bison, deer, sheep and all species combined, in all cases due to lower sensitivity values (Table IIb). Minor increases, or no changes, in PI values were noted with the remainder of the sera tested. The sensitivity and specificity values for the combined species resulted in a small decrease in sensitivity (1.0%) (Table Ic) and a small increase in specificity (0.8%) (Table Id), resulting in a net PI increase of 4.8 when the combined antigen was used, compared with the results produced by the OPS antigen (Table III). A universal cutoff for the FPA was not useful as the performance was considerably decreased (data not presented).

The Youden's plot (Fig. 1) is a graphical interpretation of the PI values of the I-ELISA. There is a 99% probability that the points within the circle near the top centre of the graph are similar. Points near the 45° line, but removed from the point of intersection (circle centre), may indicate a systematic error. Points further removed from the reference line may indicate random error. The points within the circle contain the PIs for all animals exposed to SLPS and tested with SLPS antigen except for the sheep sera (Index 16 in Table IIa). The reason for this discrepancy may be that the positive sheep sera were selected on the basis of their reactivity in a rapid agglutination test and the complement fixation test rather than on bacterial isolation. The remainder of the outlying points are the PI values from species exposed to SLPS but tested with RLPS antigen, and the species exposed to RLPS and tested with SLPS antigen.

Table IIb
Percentage sensitivity, specificity and performance index (PI) values obtained with sera from various host species (exposed or unexposed to *Brucella* sp.) using a fluorescence polarisation assay

Species	Antigen	Cutoff	Percentage sensitivity	Percentage specificity	PI
Cattle	OPS	90	100.0	100.0	200.0
SLPS	CORE	86	95.8	98.7	194.5
	OPS/CORE	85	100.0	100.0	200.0
Swine	OPS	96	98.6	98.6	197.2
SLPS	CORE	88	35.1	38.9	74.0
	OPS/CORE	97	91.9	100.0	191.9
Bison	OPS	87	99.0	96.9	195.9
SLPS	CORE	97	84.3	92.0	176.3
	OPS/CORE	110	98.0	98.8	196.8
Goats	OPS	96	98.5	100.0	198.5
SLPS	CORE	72	95.6	70.0	165.6
	OPS/CORE	88	100	100.0	200.0
Deer	OPS	97	98.4	100.0	198.4
SLPS	CORE	84	59.4	97.4	156.8
	OPS/CORE	99	92.2	100.0	192.2
Sheep	OPS	89	94.3	98.3	192.6
SLPS	CORE	89	47.1	100.0	147.1
	OPS/CORE	88	92.9	100.0	192.9
Cattle	OPS	48	100.0	100.0	200.0
RLPS	CORE	86	100.0	98.7	198.7
	OPS/CORE	85	95.0	100.0	195.0
Dogs	N/A	N/A	N/A	N/A	N/A
Sheep	OPS	89	88.2	98.3	186.5
RLPS	CORE	85	93.5	96.6	190.1
	OPS/CORE	85	96.8	98.3	195.1

Individually set cutoff values were used for each species and antigen
The antigens used were:
a) the O-polysaccharide (OPS) portion of smooth lipopolysaccharide (SLPS)
b) the CORE region of rough lipopolysaccharide (RLPS)
Both antigens derived from *Brucella* sp. and were used both individually and combined
Dog sera gave erratic results in fluorescence polarisation assay

Table III
Comparison of the overall performance indices (PI) for all species
The percentage sensitivity and specificity values for the indirect enzyme-linked immunosorbent assay (I-ELISA) (Tables Ia and Ib) and for the fluorescence polarisation assay (FPA) (Tables Ic and Id) using individually determined cutoff values, and for the I-ELISA using a universal cutoff of 10% (PI 10%P) for each antigen and their respective combinations

Test	Antigen	Percentage sensitivity	Percentage specificity	PI	PI 10%P
I-ELISA	SLPS	95.5	98.1	193.6	190.6
	RLPS	87.7	94.8	182.5	174.7
	R/SLPS	99.9	99.5	199.4	198.4
FPA	OPS	97.0	98.8	195.8	N/A
	CORE	76.9	87.0	163.9	N/A
	OPS/CORE	96.0	99.6	195.6	N/A

N/A: not applicable

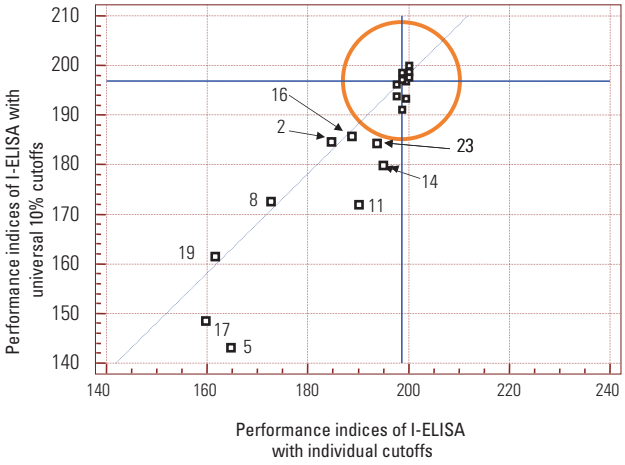

Fig. 1
Youden's plot of the indirect enzyme-linked immunosorbent assay performance index (PI) values from data using individual cutoff values compared with PI values derived from data using a universal cutoff of 10%P
The numbers in the graph refer to the index numbers in Table IIa

Discussion

The only indisputable diagnostic test for brucellosis is isolation of the causative organism from fluids or tissues of suspected hosts. However, bacteriological isolation has some major drawbacks in that it is very time consuming, expensive, operator hazardous and not amenable to mass testing. Diagnosis is therefore made by serological testing, usually of blood. Testing of milk is also possible and this topic was recently reviewed (16).

Over the years since the first serological test was described by Wright and Smith (28), a large number of tests have been developed. Until the 1970s, the test procedures relied on detection of antibody and the ability of the antibody to perform secondary functions such as agglutination, fixation of guinea pig complement or precipitation. Not all antibody isotypes were able to perform the secondary functions. This was advantageous, as the secondary performances could be used to distinguish between isotypes. Thus agglutination was generally considered to be a function of IgM, while fixation of complement was mainly a function of IgG1. Significant information regarding the disease status (chronic or acute) could be obtained with these tests. Since IgM generally disappears before IgG1 and because most cross-reacting antibody is of the IgM isotype, IgG1 antibody was used as a diagnostic marker.

As the performance of these assays in general was not satisfactory, primary binding assays were developed. The first was an I-ELISA (4), followed by radioimmunoassay (21), competitive ELISA (18), particle-concentration-counting fluorescence immunoassay (15) and FPA (19). The performance of all these tests depends on the suitability of the reagents used. For example, if a mixture of bacterial antigens and a broad-specificity detection reagent is used in the I-ELISA, lower specificity can be expected than if purified antigen and specific detection reagents are used. However, the former reagents may provide increased assay sensitivity. Thus, sensitivity and specificity are generally inversely related and, as a result, the goal of research has been to optimise both values.

There are several other problems with serological diagnosis. These include long turn-around times for results with all but rapid agglutination tests and FPA, subjectivity in interpreting the results of some tests, interference by antibody arising from vaccination, and misdiagnosis due to the presence of antibody to antigens not included in the test. Therefore, rapid, reliable and inexpensive single screening tests that perform well in detecting antibody from most susceptible species of hosts to most species of *Brucella* would be of value to the surveillance, control and eradication of this disease.

This paper describes the adaptation of two tests – an I-ELISA and an FPA, both of which are already prescribed for diagnosis of bovine brucellosis – for a single test diagnosis of rough and smooth *Brucella* sp. in several of the most important domestic livestock species. The I-ELISA and FPA are robust, perform well, are relatively inexpensive and detect antibody with high efficiency in most species tested. The I-ELISA is a laboratory test, although a dipstick format for field use has been developed (17). The FPA can be used equally well in the field and in the laboratory.

In order to adapt the tests into a single test approach, the combined antigens of S- and RLPS *Brucella* sp. were mixed in the same reaction mixture to test sera from smooth- and rough-exposed animals simultaneously. Results were compared with individually tested reagents. In order to test the multi-animal species serum sample (a broad host range), peptide-enzyme conjugate constructed from the reactive parts of proteins A and G was added to the I-ELISA. Table IIa presents the results of the I-ELISA for each host species tested with SLPS, RLPS or SLPS and RLPS antigens, using a cutoff value that maximises the PI, as well as a cutoff of 10%P, which was selected as a universal cutoff for all the I-ELISAs.

From the data, it is clear that there is a minor overall loss in PI using the universal cutoff, especially when testing bison (–2.5), deer (–1.6) and dogs (–3.6). The latter reduction was the result of one culture-positive dog giving a result between 6%P and 10%P. A universal cutoff offers

some advantage in terms of simplicity; however, the loss in PI in some cases may negate any such advantage (in the case of the dog and deer serology the loss in PI was due to decreased sensitivity, while in the case of bison, there was a loss in specificity). A compromise may be to use the universal cutoff for the major livestock species where there is no change in PI between the calculated and the universal cutoff values. Those species would include cows, swine, goats and sheep.

A universal cutoff value could not be established for the FPA without a major loss in PI. The FPA data (Table IIb) indicate that the combined antigen, the CORE and OPS, gave results similar to those obtained with the individual antigens, except in the case of cattle immunised with *B. abortus* RB51. In this case, there was a drop in sensitivity and a lesser decrease in specificity. The CORE antigen also decreased the sensitivity slightly when testing pig, bison, goat, deer and sheep sera. In all cases, the sensitivity and specificity values could be manipulated by altering the cutoff values to provide increased assay sensitivity, but with some loss of specificity. Thus by increasing the cutoff value, sensitivity could be increased at the cost of specificity. For a screening assay, high sensitivity would be desired.

Table III summarises the data from all the host species with individual and combined antigens. The I-ELISA results indicate that the PI using optimised cutoff values was reduced by 1.0 when a universal cutoff of 10%P was used.

Figure 1 is a graphical presentation of the PIs (Youden's plot), showing that the data is similar and close in magnitude for those PIs within the circle (99% coverage probability). Those points within the circle near the 45° reference line, but far from the intersection of the median lines, may indicate systematic error, while those further from the reference line may indicate random error, and the points outside the circle suggest total error. All but one of the points outside the circle are the I-ELISAs of the species exposed to SLPS but tested with RLPS, or those species exposed to RLPS but tested with SLPS. The one exception is number 16 (Table IIa), which is a sheep exposed to SLPS and tested with SLPS. Sheep exposed to RLPS and tested with SLPS, RLPS or both were within the 99% coverage probability of the circle, with no indication of systematic or random error.

An unexpected finding was the number of animals exposed to SLPS that reacted with RLPS and the CORE antigens. Similarly, a substantial number of sera from RLPS-exposed animals reacted with SLPS and OPS antigens. Presumably, the SLPS-exposed animals produce antibody to the CORE region of the SLPS molecule as well as to the OPS. In addition, it is possible that minor OPS determinants are present in *B. abortus* RB51. Similarly, RLPS-exposed animals may produce antibody that reacts with the CORE portion of SLPS. However, OPS does not contain measurable CORE region oligosaccharide and, as a result, the reaction of RLPS-exposed animals with OPS remains unexplained.

Conclusions

The development of universal assays for detection of antibody to *Brucella* sp. in sera from various hosts will allow for better control of this disease and thereby quicker eradication. While most Brucellae are reasonably species specific, some cross-species infection occurs. In particular, unexpected infection with rough *Brucella* sp. may occur where only infection with smooth *Brucella* sp. would be expected, and could therefore be missed. While the assays do not perform perfectly, they both have the advantage of electronic data assessment, allowing for shifts in cutoff values to suit each situation. For example, in areas where brucellosis has been eradicated for some time, a cutoff resulting in higher specificity values may be desired to avoid costly trace-backs. Similarly, enhanced sensitivity will allow for detection of more infected animals, particularly in the early stages of infection, leading to a more efficient control programme. It should be stressed that the single-test I-ELISA and FPA described are currently experimental tests that await complete validation. All further testing using these principles should be encouraged.

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Vers l'utilisation de tests uniques de dépistage de la brucellose

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Résumé

Le présent article décrit une méthode immuno-enzymatique indirecte (ELISA indirecte) et une épreuve de polarisation en fluorescence (FPA), chacune permettant de détecter chez plusieurs espèces d'hôtes la présence d'anticorps dirigés contre les *Brucella* en phase lisse et en phase rugueuse. La méthode ELISA indirecte utilise comme antigène un mélange de lipopolysaccharide lisse (SLPS) et de lipopolysaccharide rugueux (RLPS) et comme réactif de détection une protéine recombinante A/G conjuguée à la péroxydase de raifort. Quand on utilise des valeurs-seuil déterminées individuellement, l'épreuve ELISA indirecte à base d'antigène constitué par la combinaison SLPS/RLPS a détecté des anticorps chez un nombre légèrement supérieur d'animaux exposés au SLPS ou au RLPS que ne le permettaient les méthodes ELISA indirectes utilisant chaque antigène séparément. De même, l'épreuve employant l'association d'antigènes a détecté des anticorps chez un nombre légèrement inférieur d'animaux non exposés à *Brucella* sp. En utilisant un seuil de positivité universel de 10 % (par référence aux sérums témoins fortement positifs de chaque espèce), la valeur de l'indice de performance globale (somme, en pourcentage, de la sensibilité et de la spécificité) a diminué de 1,0 (passant de 199,4 à 198,4). Dans l'épreuve de polarisation en fluorescence, il n'était pas possible d'utiliser un seuil universel sans perte importante de performance. La valeur globale de la sensibilité de l'épreuve de polarisation en fluorescence utilisant l'antigène combiné était inférieure de 1,0 % à celle obtenue avec le polysaccharide O issu du SLPS et supérieur de 9,1 % à celle obtenue avec l'antigène de noyau (CORE) issu du RLPS. L'utilisation de l'antigène combiné a abouti à une spécificité de l'épreuve de polarisation en fluorescence légèrement supérieure (1,2 %) à celle constatée avec le polysaccharide O et très supérieure (12,6 %) à celle obtenue avec l'antigène CORE. Globalement, l'épreuve ELISA indirecte et l'épreuve de polarisation en fluorescence toutes deux employées avec des antigènes combinés étaient des techniques adaptées pour le dépistage de toutes les espèces de *Brucella* chez les espèces animales examinées.

Mots-clés

Brucellose – Diagnostic – Épreuve de polarisation en fluorescence – Méthode immuno-enzymatique indirecte – Méthode universelle – Sérologie.

Avances de las pruebas únicas para detectar la brucelosis

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Resumen

Los autores describen una prueba de inmunoabsorción enzimática indirecta (I-ELISA) y una de fluorescencia polarizada (FPA); ambas detectan la presencia de anticuerpos en varias especies huéspedes de cepas lisas y rugosas del género *Brucella*. La prueba I-ELISA emplea una combinación de lipopolisacárido liso (s-LPS) y rugoso (r-LPS) como antígeno, y una proteína recombinante A/G conjugada con peroxidasa de rábano picante como reactivo de detección. Cuando se utilizan umbrales fijados en forma individual, la prueba I-ELISA con antígeno de s-LPS y r-SPS combinados detectó anticuerpos en un número ligeramente mayor de animales expuestos al s-LPS o el r-LPS que las técnicas I-ELISA que recurren a cada uno de esos antígenos por separado. Del mismo modo, la prueba que emplea el antígeno combinado detectó anticuerpos en un número ligeramente inferior de animales que no habían estado expuestos a *Brucella* sp. Cuando se utilizó un umbral de positividad universal correspondiente al 10% (respecto a los sueros controles positivos fuertes de cada especie), el valor del índice de eficiencia global (es decir, la suma de los índices de sensibilidad y de especificidad) disminuyó 1,0 (de 199,4 a 198,4). En cambio, en la prueba FPA no fue posible aplicar un umbral general sin que se perdiese buena parte de la eficiencia. El valor de la sensibilidad global de la prueba FPA realizada con el antígeno combinado era 1,0% más bajo que el obtenido utilizando el polisacárido O del s-LPS y 9,1% más elevado que el obtenido con el antígeno central (CORE) del r-LPS. Cuando se utilizó el antígeno combinado, la especificidad de la prueba FPA era ligeramente superior (1,2%) que con el polisacárido O exclusivamente, y considerablemente superior (12,6%) que al emplear el antígeno central. En conclusión, ambas pruebas con antígenos combinados dieron resultados satisfactorios para detectar todas las especies de *Brucella* en las especies animales examinadas.

Palabras clave

Brucelosis – Diagnóstico – Prueba de fluorescencia polarizada – Prueba de inmunoabsorción enzimática indirecta – Prueba universal – Serología.

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