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Research paper

A new homogeneous assay for high throughput serological diagnosis of brucellosis in ruminants

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ABSTRACT

The control and eradication of brucellosis is highly desirable but heavily resource intensive as high throughput serological testing is required. The aim of this study was to meet the needs of high throughput screening laboratories involved in this process through the development of a new assay. An existing cELISA used for the serodiagnosis of brucellosis in ruminants was converted to an AlphaLISA homogenous proximity based assay. This assay requires no separation steps and can be performed in low volume microtitre format. The *Brucella* AlphaLISA was validated on a panel of bovine, ovine and caprine sera from infected and uninfected animals. The diagnostic sensitivities (>96%) and specificities (>98%) obtained compared well to those from cELISA, iELISA and FPA performed on the same samples. The AlphaLISA met the testing criteria set for ELISAs as defined by the OIEELISA standards and had an analytical sensitivity similar to that of the parent cELISA. The method was also used on a small panel of serum samples from cattle that were experimentally infected with *Yersinia enterocolitica* O:9. Some false positive reactions were obtained as was also the case with results from FPA, iELISA, cELISA, CFT and SAT. Despite this, the methodological advantages of the AlphaLISA mean that this assay is well suited to high throughput serodiagnosis. This report is the first description of the use of AlphaLISA to detect pathogen specific antibodies. Furthermore, the relative ease with which the cELISA was converted to this platform indicates that this technology is ready to meet the high throughput testing requirements for the diagnosis of many other diseases.

1. Introduction

Brucellosis is a global zoonotic disease which causes considerable animal and human health problems as well as huge economic costs. The disease is caused by bacteria of the genus *Brucella* which belong to the α-2 subdivision of Proteobacteria. The genus consists of six classical species, *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Brucella ovis*, *Brucella canis*, and *Brucella neotomae* plus more recently discovered strains from marine mammals. Of the *Brucella* species, *B. abortus*, *B. melitensis* and *B. suis* are of principal human health and economic importance. These species have smooth lipopolysaccharide (LPS) which is considered a major virulence factor of disease (Porte et al., 2003) whereas *B. ovis* and *B. canis* have rough LPS.

The economic burden of effective brucellosis surveillance is high. For example, in order to maintain brucellosis free status in Great Britain approximately 1 million bovine serum samples were examined by iELISA (indirect ELISA) annually. In recent years the Republic of Ireland has performed approximately five

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million blood tests for brucellosis per annum. It is clear that high throughput serological testing is a key tool in the control of brucellosis.

The OIE prescribed and alternative serological tests for brucellosis due to infection with smooth strains rely largely upon the measurement of the hosts generated antibody response to the O-antigen of the smooth LPS (Nielsen and Ewalt, 2004). Classical tests include the Rose Bengal test (RBT), the complement fixation test (CFT) and the serum agglutination test (SAT) all of which employ a whole cell antigen as the key diagnostic reagent. More contemporary techniques such as the indirect (i) ELISA, competitive (c) ELISA and the fluorescent polarisation assay (FPA) employ purified LPS or O-antigen as the diagnostic reagent. The immunodominance of the LPS O-antigen is the basis for the generally good sensitivity of these assays. However, the use of this antigen can lead to false positive serological results (FPSRs) when animals are infected with bacteria possessing O-antigen of similar structure (Corbel, 1985) such as *Yersinia enterocolitica* O:9.

ELISA tests are readily amenable to high throughput testing due to the standardised nature of the technology and reagents. This allows for many efficiency savings including the introduction of automation (McGiven et al., 2007). Despite the advantages of ELISA over the classical tests in this regard, ELISAs still require several steps to complete including separation steps. Although these steps can be automated they are a vital part of the assay and are a frequent source of imprecision, error and mechanical breakdown. Assays which have the advantages of the ELISA, such as a 96 well format, objective assessment and good sensitivity and specificity, but which reduce the burden of work and opportunity for error are clearly desirable.

The AlphaLISA is a homogeneous proximity based assay (Ullman et al., 1994) whereby two types of 200 nm diameter neutral density beads, a donor and an acceptor, are drawn together by the biological interaction of their conjugates. Upon laser excitation at 680 nm, a photosensitiser in the donor bead converts adjacent ambient oxygen to an excited form of singlet oxygen. The singlet oxygen molecules diffuse across to react with thioxene derivatives in the acceptor bead that ultimately emit light at 520–620 nm. The singlet oxygen has a half life of approximately 4 μs and can diffuse approximately 200 nm from the donor bead during this period. If an acceptor bead is within this range, then light will be emitted. The sensitivity of the technology is enhanced by the anti-Stokes shift effect where the emitted light is of a lower wavelength than the absorbed light. Non-specific matrix fluorescence will be of a higher wavelength and will not cause signal interference. This method has also been described in the literature as a Luminescence Oxygen Channelling Immunoassay (LOCI).

The aim of this study was to develop a new homogenous serological assay for the diagnosis of brucellosis by binding the active biological components used in the cELISA (competitive ELISA) for brucellosis (Stack et al., 1999) to AlphaLISA beads. This includes the validation of this new assay against existing serological methods using serum from *Brucella* infected and non-infected ruminants as well as sera from cattle experimentally infected with *B. abortus* or *Y. enterocolitica* O:9. The latter being used to assess the potential impact of FPSRs on the *Brucella* AlphaLISA.

2. Materials and methods

2.1. AlphaLISA

The AlphaLISA acceptor beads (Perkin Elmer No. 6762003) were conjugated with BM40 monoclonal antibody (Mab) (Greiser-Wilke et al., 1985). These beads were supplied with an aldehyde functional group on their surface to facilitate conjugation which was performed as follows. Two vials of beads, each containing 1 mg were vortexed for 30 s to suspend the beads, and the contents pooled. These beads were then pelleted at the bottom of a 2 ml microfugetube by centrifugation at 15 rcf for 4 min and the supernatant (storage buffer) discarded. The beads were then resuspended in 100 μl PBS-T (Phosphate Buffered Saline with 0.5% Tween 20) and then pelleted as before with the supernatant discarded. The beads were resuspended in 400 μl PBS-T containing 0.2 mg of BM40 Mab and 0.5 mg of sodium cyanoborohydride (Sigma No. 71435) and incubated in the dark at 37°c for 48 h. After this period, 20 μl of a 0.3 M Glycine (Sigma No. 241261) solution was added and the reagents incubated for a further 1 h under the same conditions. The beads were then washed twice by centrifugation as described above and resuspended to a final volume of 400 μl and final concentration of 5 mg/ml in PBS-T.

The antigen used in the cELISA is smooth LPS antigen derived from *B. melitensis* strain 16 M by phenol extraction (Westphal et al., 1952). In order to conjugate this antigen to AlphaLISA beads it was modified by labelling with EZ-Link Biotin-LC-Hydrazide™ (Pierce) following the manufacturers instructions. Briefly, the LPS was oxidised by incubation (in the dark) in a cold solution of sodium meta-periodate (Sigma No. 311448) in sodium acetate buffer (pH 5.5). After a 30 min incubation the excess periodate was removed by buffer exchange using PBS and then the biotin hydrazide, dissolved in Dimethyl sulfoxide (Sigma No. D-5879), was added. The LPS and Biotin-LC-Hydrazide were incubated at room temperature for 2 h before excess biotin was removed by desalting and buffer exchange using Zebra™ Desalt Spin Columns (Pierce) and following the manufacturer's instructions. The biotinylated LPS (bLPS) antigen was then bound to streptavidin-coated donor beads (Perkin Elmer No. 6760002S) as part of the assay process.

The AlphaLISA was performed in 96 well microtitre plates (white opaque OptiPlate™ from Perkin Elmer) and initial optimisation was performed at a final test volume of 50 μl per well and a final concentration of acceptor and donor beads of 10 μg/ml and 40 μg/ml respectively. The bLPS was titrated against the described concentration of donor and acceptor beads. The concentration of antigen that generated the highest level of emitted light from the acceptor beads was chosen as the final working strength. The optimal test and control serum concentration was chosen by titration against the bead and antigen concentrations described above. Four controls were used, a caprine high titre positive control, an ovine low titre positive control, an ovine negative control, and a bead conjugation control (10 μl of test buffer added instead of serum). The concentration of sera that produced the biggest difference between the emitted light from a well containing negative sera and a well containing a weak positive sample was chosen as the final test dilution.

To perform the test, acceptor beads were prediluted in test buffer (PBS-T) from the stock of acceptor beads (after vortexing for 10 s to resuspend the beads) to a concentration of 33.3 μg/ml. The bLPS was prediluted to five times the concentration of the final working strength and test sera and controls were prediluted 1/30 in test buffer. The acceptor beads and the antigen were then added to the wells on the plate in 15 and 10 μl volumes respectively. Then 10 μl of test/control sera were added to the respective wells on the plate using the pipetting action to mix the reagents. The test plate was then sealed by the application of an adhesive film and the plate was incubated in the dark, without any further mixing or shaking, for 1 h. Prior to the completion of this time period, a predilution of the donor beads was prepared by diluting the stock solution to a concentration of 133.3 μg/ml. This is stable so it can be prepared earlier. After 1 h, the film covering the test plate was removed and 15 μl of the donor bead predilution was added to each well and mixed as before. The test plate, now containing 50 μl of reagents per well, was covered with adhesive film and incubated as before. During the addition of donor beads care was taken to avoid any prolonged direct exposure to bright fluorescent lighting.

After completion of the second 1 h incubation period, the test plate was read on an EnVision plate reader from Perkin Elmer using standard factory settings to measure the degree of fluorescence produced by the acceptor beads. The test result for each sample and control was expressed as the percentage inhibition (PI) of the bead conjugation control. The results of the plate were deemed acceptable if the control samples produced data within defined limits. Four controls were used, as described above. This method was used to test the serum validation panel described below.

The analytical sensitivity of the AlphaLISA and cELISA was determined by measuring the effect of the introduction of free BM40 as a replacement for test serum. The BM40 was added to the test wells at a final concentrations ranging from 128 to 0.0625 nM for the AlphaLISA and cELISA. The sensitivity of the AlphaLISA was also assessed using the OIEELISA$_{SP}$SS and OIEELISA$_{N}$SS (Office International des Epizooties ELISA Strong Positive and Negative Standard Sera) samples that are used to standardise ELISAs for the diagnosis of bovine brucellosis (Nielsen and Ewalt, 2004; Bryne 2002). The OIEELISA$_{SP}$SS was tested using neat to 1/64 double dilutions in negative bovine serum and the OIEELISA$_{N}$SS was only used neat, each was then subsequently diluted 1/150 in the test well in accordance with the developed AlphaLISA test protocol.

2.2. AlphaLISA modifications

After validation of the AlphaLISA, two modifications to the protocol were briefly assessed for their potential to improve the method. The first was to add the donor beads immediately after the addition of serum followed by a single 2 h incubation. The effects of these changes were assessed against negative serum, positive serum and conjugate controls as well as dilutions of the OIEELISA$_{SP}$SS. The second was the addition of the metal chelating agents Deferoxamine Mesylate (DFO) (Sigma No. D9533) and EDTA (Sigma No. E6758) to the test buffer at final concentrations of 32 to 0.125 nM and 128 mM to 0.5 nM respectively. The effects of these changes were assessed against negative, positive and conjugate controls as well as haemolysed cattle samples.

2.3. CFT, SAT and ELISAs

Both iELISA and cELISA were performed as described previously (McGiven et al., 2003). The determination of a positive or negative result for serum samples tested followed the interpretation for each ELISA method used. The CFT and the SAT were performed as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Nielsen and Ewalt, 2004).

2.4. FPA

The FPA was performed using antigen supplied by Diachemix™ and in accordance with their kit instructions. The microtitre plate method was used whereby serum is tested at a 1/10 dilution with 180 μl FPA buffer per well. A Tecan GENios Pro was used to measure fluorescence polarisation. An initial reading of the buffer and serum was taken to measure the background polarised fluorescence of the sample. Then 10 μl of the test antigen was added and the plate was incubated at room temperature for 2 min on a rotary shaker. A second reading was taken which was 'blanked' using the data from the first read, and from this the value of each sample in millipolarisation (mP) units was calculated (Nielsen and Gall, 2001). Positive/borderline/negative interpretations were made according to the kit instructions, however in order to compare the FPA to other methods two summary data sets, alternately classifying "borderline" samples as positive or negative, were prepared.

2.5. Serum validation panel

To assess the diagnostic specificity of the AlphaLISA single serum samples from 240 randomly selected cattle from Great Britain were collected. In addition, serum samples from 160 randomly selected sheep and goats were also collected. To assess the diagnostic sensitivity of the assay, single serum samples from 27 cattle and 15 sheep and goats were tested. Of the bovine samples: eight were from animals confirmed as infected by culture and from natural infections, two were from culture positive animals experimentally infected with *B. abortus* strain 544, nine were from culture positive animals although biotyping had not ruled out the possibility that the recovered *Brucella* were the vaccine strain S19, and a further seven were from serologically positive (by CFT and SAT) animals from a culturally confirmed outbreak of brucellosis. Of the 15 sheep and goat samples: two were from animals confirmed as infected by culture and from natural infections, five were from culture positive animals from experimental infection with *B. melitensis*, and the remaining nine animals were serologically positive (by CFT and SAT) animals from a culturally confirmed outbreak of brucellosis. This validation panel was also tested using iELISA, cELISA and FPA.

Some of the 240 non-*Brucella* infected bovine samples demonstrated a high degree of haemolysis. These were identified quantitatively by diluting all the samples 1/10 in

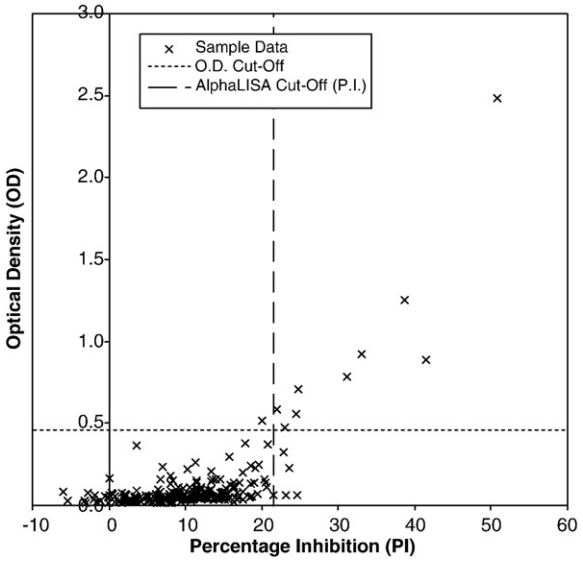

Fig. 1. AlphaLISA test results, expressed as PI of the bead control, against OD of samples diluted 1 in 10 in PBS (measured at 450 nm) for 240 samples from *Brucella* uninfected cattle. The horizontal dashed line represents the cut-off for the OD values. Samples above this line have been excluded from further analysis. The vertical dashed line represents the positive/negative cut-off for the AlphaLISA as determined from the TW-ROC analysis (Fig. 2).

PBS to a total volume of 100 µl in a standard clear 96 well microtitre plate (Nunc). The plate was then measured at an optical density (OD) of 450 nm.

2.6. Experimental infections

The AlphaLISA was also assessed against a panel of serum from cattle experimentally infected with either *B. abortus* or *Y. enterocolitica* O:9. Two groups of four Holstein/Fresian-cross cattle were infected independently with either *B. abortus* strain 544 (10^9 colony forming units) via the ocular route, or *Y. enterocolitica* O:9 (10^{12} colony forming units) orally on 4 occasions on alternate days. The two animal groups were then kept apart to prevent cross infection. All cattle were confirmed free of both *Yersinia* and *Brucella* prior to experimental infection and microbiological investigations confirmed that subsequent infection had taken place. Serum from each animal was tested by AlphaLISA at 3, 7, 16, 24, and 53 weeks post infection. This selection of sera was also tested by CFT, SAT, iELISA, cELISA and FPA. All animal procedures were conducted in accordance with the United Kingdom Animal (Scientific Procedures) Act 1986.

2.7. Data analysis

Regression analysis was performed using the method of 'least squares' and the significance testing of the regression coefficient was performed by ANOVA. The results from the AlphaLISA, expressed as the PI of the conjugate control, were used to plot Two Way Receiver Operator Curves (TW-ROC) for bovine and sheep and goat samples (Greiner et al., 1995). These curves were used to find the positive/negative cut-off for the test in both species that maximised the diagnostic sensitivity (DSn) and diagnostic specificity (DSp) of the assay. Confidence intervals for the DSn and DSp were calculated using Normal Distribution models (Jacobson, 1998). Comparison of dichotomous paired data from the AlphaLISA and the FPA was performed by McNemar's test. All analyses were performed using Microsoft Excel software.

3. Results

3.1. Results from testing the validation panel

A scatterplot of the AlphaLISA results from the 240 serum samples from non-infected cattle against their OD (a measure

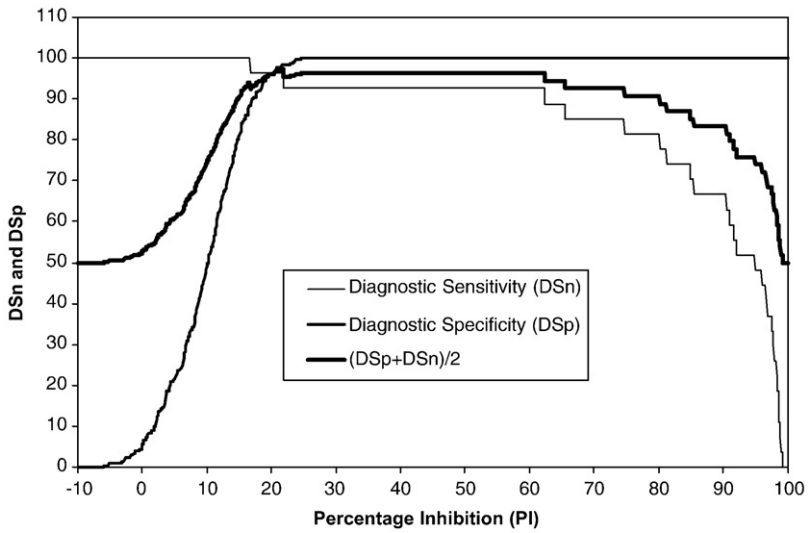

Fig. 2. TW-ROC of AlphaLISA on 230 samples from *Brucella* uninfected cattle and 27 samples from *Brucella* infected cattle. The thin and medium weighted lines show the DSn and DSp percentages respectively against test positive/negative cut-offs set along a continuous PI scale. The thickest line shows the average DSn and DSp value at each PI.

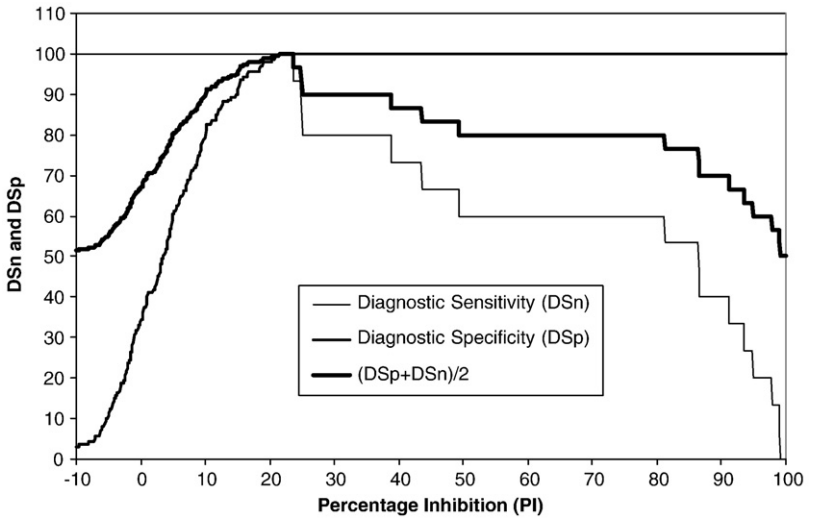

Fig. 3. TW-ROC of AlphaLISA on 160 samples from *Brucella* uninfected sheep and goats and 15 samples from *Brucella* infected sheep and goats. The thin and medium weighted lines show the DSn and DSp percentages respectively against test positive/negative cut-offs set along a continuous PI scale. The thickest line shows the average DSn and DSp value at each PI.

of the degree of haemolysis) shows that the majority of the samples have results of less than 20% inhibition of the conjugate control (Fig. 1). There is also a positive relationship between the OD of the sample and its PI, especially at the higher PI values. Linear regression analysis of the data confirms this positive relationship (regression coefficient of 0.013) and ANOVA confirms that this positive relationship is significant ($P<0.001$). Visually, the samples with high ODs are dark red in colour and would be unsuitable for many types of test method and are samples that have been poorly stored or stored with red blood cells for too long before testing. The relatively large number of outlying data prevents the effective modelling of the distribution. The ten samples with the highest OD represent 2.4% of the total number and each is greater than the average OD plus 1.645 times the standard deviation. This places them in highest 5% of a normal distribution model based on the observed mean and standard deviation (although this model is not very accurate). These ten samples have an average PI result of 29.05%. The average for all 240 samples is 10.66%, the average excluding these ten samples is 9.78%. The results for these ten, most haemolysed, outlying samples were excluded from further analysis.

A TW-ROC of the results from the remaining 230 bovine samples tested (Fig. 2) shows the calculated DSn and DSp for the assay against potential positive/negative cut-off values situated along the PI scale. The maximal average of the DSp and DSn value is (97.28%) at a cut-off of 21.5 PI, with DSp=98.26% and DSn=96.30%. All the ovine and caprine samples collected were of good quality with no visual evidence of haemolysis so all the data was included in the TW-ROC analysis (Fig. 3). There is a small interval, between 21.5 and 23.6 PI where the DSp and DSn value are both 100%. This interval includes the optimal cut-off value for the bovine samples.

A summary of all test results (Table 1) shows that the DSn of the AlphaLISA is equal to that of the ELISAs, higher than the FPA with the ovine and caprine samples but lower with the bovine samples although there are no significant differences (95% percent confidence intervals for DSp or DSn estimates are shown in brackets). The DSp of the AlphaLISA is equal to that of the ELISAs with the ovine and caprine samples but lower with the bovine samples although this difference is not significant. For all species, the AlphaLISA showed superior specificity than the FPA. This difference was not significant in the case of the ovine and caprine samples as the confidence

Table 1
Summary of diagnostic sensitivity (DSn) and specificity (DSp) values for each test method used on the AlphaLISA validation panel

Cattle	AlphaLISA	FPA (bl+)	FPA (bl−)	iELISA	cELISA
DSn (n=27)	96.30 (±7.12)	100.00	100.00	96.30 (±7.12)	96.30 (±7.12)
DSp (n=230)	98.26 (±1.69)	95.22 (±2.76)	85.22 (±4.59)	100.00	100.00
Cattle	AlphaLISA	FPA (bl+)	FPA (bl−)	iELISA	cELISA
DSn (n=15)	100.00	93.30 (±12.65)		100.00	100.00
DSp (n=160)	100.00	98.75 (±1.72)		100.00	100.00

For each test type the DSn and DSp are shown with the number of animals of each category from which a serum sample was tested displayed as 'n'. The values in brackets show the 95% confidence interval for the DSn and DSp. For the FPA "(bl+)" indicates that in this analysis the "borderline" results were interpreted as positive, the "(bl−)" indicates that these samples were interpreted as negative in this analysis.

Table 2
Test results for serum taken from eight cattle experimentally infected with either *B. abortus* or *Y. enterocolitica* O:9

Infection and animal ID	Week no. post infection					Week no. post infection				
	3	7	16	24	53	3	7	16	24	53
	AlphaLISA (PI of bead control)					CFT (ICFTUs)				
Brucella 1	63.5	66.8	87.8	76.6	86.5	133.2	372.8	851.2	170.2	1702.4
Brucella 2	56.6	59.4	43.1	33.1	23.4	160.0	133.2	80.0	46.6	0.0
Brucella 3	38.8	64.3	67.0	49.8	24.1	93.2	266.4	80.0	53.2	0.0
Brucella 4	83.3	88.4	81.4	68.7	51.2	532.8	1702.4	425.6	266.4	33.3
Yersinia 1	68.2	34.9	24.6	20.9	21.0	80.0	33.3	0.0	0.0	0.0
Yersinia 2	55.2	45.8	38.7	28.5	20.6	372.8	66.6	20.0	10.0	0.0
Yersinia 3	61.0	24.6	26.7	17.4	14.4	93.2	66.6	8.3	0.0	0.0
Yersinia 4	60.0	20.7	11.0	13.6	12.4	46.6	20.0	0.0	0.0	0.0
	cELISA (PI of conjugate control)					SAT (IUs)				
Brucella 1	74	86	91	88	93	410.5	410.5	328.0	410.5	1148.5
Brucella 2	54	77	73	71	29	492.5	205.0	51.5	31.0	20.5
Brucella 3	74	89	85	81	18	205.0	143.5	61.5	31.0	18.0
Brucella 4	86	92	88	82	53	1641.0	492.5	123.0	72.0	20.5
Yersinia 1	65	65	41	33	8	492.5	205.0	51.5	31.0	20.5
Yersinia 2	77	83	73	62	28	410.5	123.0	20.5	15.5	0.0
Yersinia 3	67	46	48	37	22	246.0	61.5	18.0	13.0	0.0
Yersinia 4	50	44	42	9	5	205.0	61.5	25.5	15.5	13.0
	FPA (mPs above negative control)					iELISA (percentage of cut-off control)				
Brucella 1	41.5	151.1	183.4	180.0	184.7	122.4	152.6	153.4	155.0	149.7
Brucella 2	48.7	132.7	81.5	20.5	7.4	90.5	129.4	137.7	138.1	112.3
Brucella 3	28.4	122.7	67.8	37.7	8.0	117.4	138.4	149.7	147.5	125.0
Brucella 4	100.3	159.1	126.4	87.6	34.0	139.3	142.2	157.5	154.1	132.3
Yersinia 1	30.1	−0.6	6.6	−5.2	7.4	101.8	55.3	90.6	81.0	97.0
Yersinia 2	106.7	87.6	60.1	18.8[bl]	43.9	144.4	143.8	136.6	127.5	104.5
Yersinia 3	55.6	36.2	−0.4	−1.6	−0.4	112.8	121.2	105.2	106.3	103.7
Yersinia 4	23.5	24.0	6.6	−2.8	0.0	113.0	124.8	91.8	81.2	79.1

Test results on serum taken from four *B. abortus* and four *Y. enterocolitica* O:9 infected cattle at five different time points post infection using AlphaLISA (Cut-Off 21.5 PI), cELISA (Cut-Off 40 PI), FPA (Cut-Off 20 mP), CFT (Cut-Off 20 International Complement Fixation Test Units (ICFTUs)), SAT (Cut-Off 30 International Units (IUs)) and iELISA (Cut-Off 100 Percent of Cut-Off Control). The results marked in grey cells show a positive result for that test. The result marked with 'bl' indicates that the test interpretation was 'borderline' (FPA only).

interval for the FPA DSp overlaps with the DSp for the AlphaLISA. This was not the case for the bovine samples, but McNemar's test shows that there is no significant difference between the DSp of the AlphaLISA and FPA (P=0.12) when "borderline" samples were classified as negative. When these samples were classified as positive, the confidence intervals

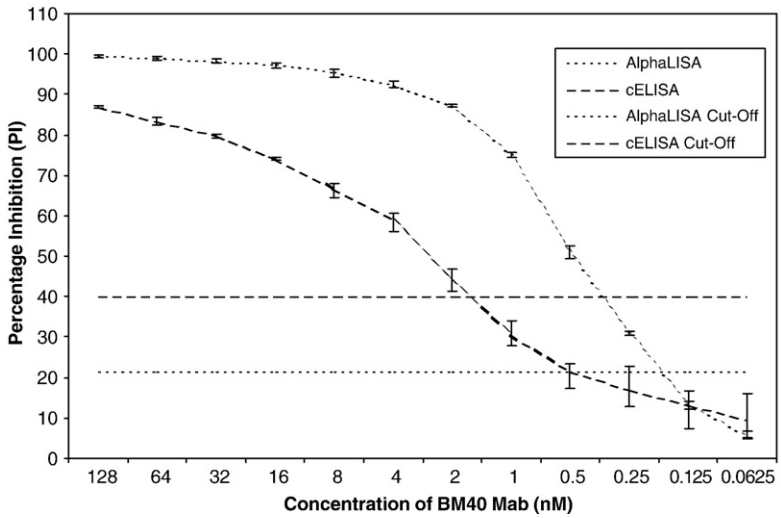

Fig. 4. PI of conjugate control in the cELISA and bead control in the AlphaLISA against nM concentration of unlabeled BM40 Mab. The lower horizontal dashed line represents the positive/negative cut-off for the AlphaLISA established by TW-ROC (Figs. 2, 3.) The higher horizontal dashed line represents the standard positive/negative cut-off used in serological testing by cELISA. The error bars represent the maximum and minimum results from three replicates for the AlphaLISA and two for the cELISA.

do not overlap demonstrating that the AlphaLISA has a significantly higher DSp than the FPA in this case.

3.2. Analytical sensitivity

The analytical sensitivity results for the AlphaLISA and cELISA are shown by the decrease in PI of the conjugate control in both the AlphaLISA and the cELISA as the concentration of unlabelled BM40 decreases (Fig. 4). The data demonstrates that the AlphaLISA can effectively discriminate between a 0.125 and 0.0625 nM concentration of unlabelled BM40 and has a half maximal inhibitory concentration (IC_{50}) of approximately 0.5 nM. In the cELISA the lowest concentration of BM40 at which discrimination between adjacent values is reliable is 1 nM. The IC_{50} value for the cELISA is between 4 and 2 nM. In this context the AlphaLISA is 4 to 8 times more analytically sensitive than the cELISA. However, the dynamic range of the cELISA appears to be greater than that of the AlphaLISA. Once the latter has had approximately 4 nM of BM40 added, the addition of more BM40 makes little difference to the test result. These results show that the positive/negative cut-off for each test lies on the linear portion of the respective curves. This cut-off corresponds to an unlabelled BM40 concentration of approximately 2 nM in the cELISA and between 0.25 and 0.125 nM for the AlphaLISA showing that, in this context, the AlphaLISA is between 8 and 16 times more sensitive. However these values are offset by the serum dilution factor for the cELISA which is 1/6 whereas for the AlphaLISA this is 25 times greater at 1/150. The magnitude of the error bars (Fig. 4) demonstrates that the AlphaLISA is more precise than the cELISA. The average CV of the three replicates for each Mab concentration is 3.3% for the AlphaLISA and 9.5% for the cELISA.

The AlphaLISA results for the dilutions prepared from the $OIEELISA_{SP}SS$ (Fig. 5) show that a 1/16 predilution of the standard in negative serum is consistently positive when tested at working strength (1/150). The error bars represent the maximum and minimum result from three tests. The average CV of these replicates was 1.8%. The test cut-off, 21.5%, is shown by the dashed line. The $OIEELISA_{N}SS$ is consistently negative when tested neat.

3.3. Samples from experimentally infected animals

Out of 20 samples from the *B. abortus* infected animals the number of positive results from each test were as follows (Table 2): AlphaLISA (20) > iELISA (19) > cELISA = CFT = FPA (18) > SAT (17). From the 20 samples from the *Y. enterocolitica* O:9 infected animals the number of negative results from each test were: CFT (11) > FPA = SAT (10) > AlphaLISA (9) > iELISA = cELISA (7), although the FPA did produce one 'borderline' result that has not been included in either total. The exact pattern of the results and how they are distributed against individual animals and time post infection are shown (Table 2). The serum from animal *Brucella* 1 has a consistently strong serological response in all tests and animal *Yersinia* 2 produces the most false positive results overall. All tests generate positive results from serum taken three weeks post infection in all four *Y. enterocolitica* O:9 infected animals.

3.4. AlphaLISA modifications

The results from tests performed with simultaneous bead addition using positive and negative control sera and serial dilutions of the $OIEELISA_{SP}SS$ demonstrated a clear graduated distinction between the positive and negative sera (data not shown). However, the results indicate that a positive/negative cut-off for such a method would be different from the standard method where the beads are added at different time points.

The results from the tests conducted with DFO and EDTA added to the test buffer did not show improvement in diagnostic performance (data not shown). Both chelates increased the inhibition of the fluorescent signal from the beads for all sample types tested, haemolysed negative, haemolysed

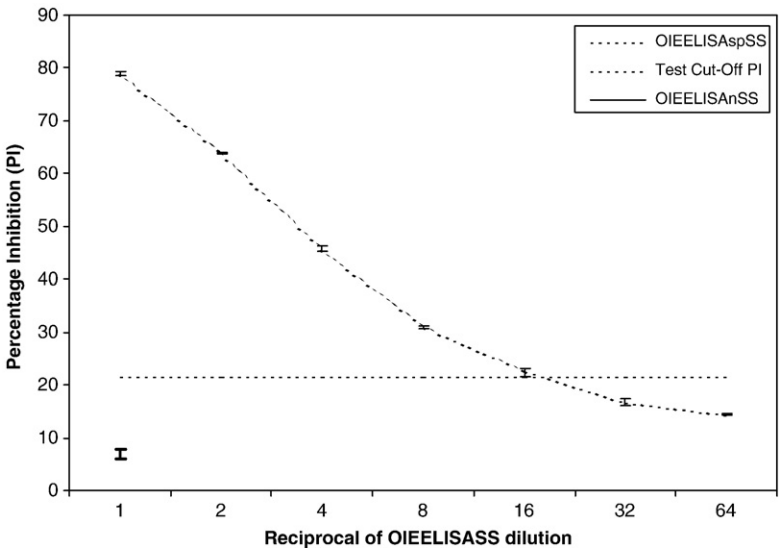

Fig. 5. AlphaLISA results expressed as PI for serial dilutions of the $OIEELISA_{SP}SS$ in negative serum and a the $OIEELISA_{N}SS$ neat, subsequently diluted 1/150 as per the test protocol. The horizontal dashed line represents the positive/negative cut-off for the AlphaLISA as established by TW-ROC (Figs. 2, 3). The error bars represent the maximum and minimum values from three replicates at each dilution.

positive, negative and positive controls. This was especially prominent with the DFO. There was also no proportional reduction in the difference between the results from the negative control and the haemolysed negative sample.

4. Discussion

The aim of this project was to develop a new homogeneous assay, based on the cELISA, for the serological diagnosis of brucellosis in ruminants and perform a small scale validation exercise. The underlying bio-molecular principle of the assay remains the same as in the cELISA in that competition by serum antibodies for antigen epitopes will result in reduced Mab binding. In the cELISA this manifested by a reduced OD as the enzyme conjugated Mab is inhibited from binding. In the AlphaLISA, the fluorescent signal is inhibited as there is a reduced level of the Mab to antigen binding that forms the bridge between the donor and acceptor beads. The AlphaLISA beads supplied by Perkin Elmer possess generic biological properties that enable, with minimal manipulation, and using standardised protocols, binding of the specific Mab and LPS antigen to the appropriate bead type. The optimisation of test incubation time was quick and simple to perform. The conversion of the cELISA to an AlphaLISA homogenous platform was rapid and straightforward. To the knowledge of the authors this is the first time that AlphaLISA (or LOCI) has been used to detect pathogen specific antibodies.

The TW-ROC analysis of the bovine samples (Fig. 2) shows the optimal point to position the test cut-off in order to maximise the desired diagnostic properties. In this case 21.5 PI gave the best combination of DSn and DSP. Most of the samples from the infected cattle were of relatively high titre as shown by the maintenance of the DSn above 90% up until just above 60 PI. The TW-ROC for the ovine and caprine samples (Fig. 3) shows a point of overlap of the DSn and DSp curves at 100% demonstrating that with the AlphaLISA, complete separation of the samples from infected and non-infected sources could be achieved at the appropriate cut-off. In this case 21.5 PI was also suitable. The DSn decreases rapidly after the cut-off point indicating that some of the positive samples are of relatively low titre.

The summary of the DSn and DSp results (Table 1) shows that the diagnostic performance of the AlphaLISA is similar to that of more established methods. The ELISAs were both marginally superior owing to improved diagnostic specificity although this was not statistically significant. There is no significant statistical difference between the FPA and the AlphaLISA unless “borderline” FPA results are considered negative. In this case the AlphaLISA is statistically superior. Therefore these results demonstrate that the *Brucella* AlphaLISA has the potential to match or possibly exceed the performance of the established techniques presented here.

The analysis of analytical sensitivity shows that the *Brucella* AlphaLISA is capable of subnanomolar detection of antibodies as demonstrated by the IC_{50} value of 0.5 nM. This is superior to the performance of the cELISA although when this is extrapolated to a serological context, the difference in testing dilutions of serum indicates that the two tests have similar analytical performance in this situation. The reproducibility of the test is excellent with an average CV between replicates of 3.3%. Compared to the ELISAs, the elimination of plate coating and separation steps in the AlphaLISA reduces the opportunity for error and improves precision.

The performance of the AlphaLISA was measured against the OIEELISA standards that are used to standardise the performance of ELISA methods for the serological diagnosis of brucellosis (Nielsen and Ewalt, 2004; Bryne, 2002). These results (Fig. 5) show that the AlphaLISA passes the European Commission (EC) criteria set for ELISAs: the 1/16 predilution (in negative sera) of the $OIEELISA_{SP}SS$ was positive and the 1/64 predilution (in negative sera) was negative. The $OIEELISA_{N}SS$ was also negative, as required. The results also meet the OIE criteria for ELISAs. This is further evidence to support the strong performance of the AlphaLISA and demonstrates that the OIEELISA sera could be appropriate to standardise this test. Once more, the data showed the good reproducibility of the method with an average CV of the replicates of just 1.8%.

Unfortunately some of the bovine samples obtained for validation were of sub-standard quality and had haemolysed. The degree of haemolysis was crudely measured by OD measurement and this data was used to show that the PI result from the AlphaLISA is increased when used with haemolysed samples. In order to prevent this affecting the validation the poorest quality samples were excluded from the analysis. In terms of serological cross reactivity this is a non-biased exclusion so it did not detract from the quality of the remaining data. Whereas both the iELISA and the cELISA classified the ten excluded samples from the original number of 240 as negative, the AlphaLISA and FPA classified nine and eight of these as positive respectively. The suitability of the cELISA for testing poor quality samples has already been demonstrated (Stack et al., 1999).

It was hypothesised that the high levels of iron in the serum due to haemolysis could have an inhibitory impact on the AlphaLISA due to the reaction of the singlet oxygen molecules released from the donor beads with the iron before it diffused across to the acceptor beads. The addition of metal chelates to the test buffer did not show any improvement in results on the haemolysed serum. The ovine and caprine samples that were collected were in a far more suitable, and typical, condition. This is possibly one reason why the AlphaLISA results from these sera were superior to those from the cattle. Re-validating on bovine sera of superior quality may yield improved results.

The good diagnostic performance of the AlphaLISA, its small volume microtitre format and its homogeneous nature make this method ideally suited to high throughput testing. In such scenarios, the elimination of any steps within the method could add significantly to the efficiency of the system. To this end, a modification to the validated protocol — the addition of both beads at the same step, was assessed. The results showed differing PI values for the same serum for the two methods, but both methods provided a clear distinction between the control sera demonstrating that the simpler, modified method has the potential to be used for diagnosis. The assessment of this method was not part of the initial project as the addition of the two bead types at the same stage was not recommended by the manufacturer, yet in this analysis it clearly shows promise.

The infection of ruminants with bacteria that possess epitopes of similar LPS O-antigen structure to that of *Brucella* is suspected to be the cause of many FPSRs in serological assays that are based on the O-antigen (Gerbier et al., 1997). To assess the impact of infection with such cross-reactive bacteria on the AlphaLISA samples from cattle experimentally infected with *B. abortus* and

Y. enterocolitica O:9 were tested and the results compared to those from other serological techniques. The results show that the AlphaLISA has excellent sensitivity but, like all the other methods assessed, remains prone to false positive reactions caused by *Y. enterocolitica* O:9. As a homogeneous homologue of the cELISA, such results were not unexpected as the underlying basis for the biological interaction is the same. Although the results for both the cELISA and the AlphaLISA were disappointing in this regard, it was of interest to observe that the AlphaLISA classified less false positive samples whilst remaining more sensitive to samples from *Brucella* infected animals.

A number of assays have been published using non-smooth LPS derived antigens for diagnosis, e.g. rough LPS and proteins (Nielsen et al., 2005; Muñoz et al., 2005), but none have been able to match the values for diagnostic sensitivity and specificity for the ELISAs and FPA — which are typically much in excess of 95% (McGiven et al., 2003). However, these assays may have a role to play in the resolution of FPSRs. Non-serological assays such as the IFNg (Kittelberger et al., 1997) and skin test (Godfroid et al., 2002) may also provide some resolution of serological cross reaction. However, to use either as a screening surveillance test would be far more costly and less effective than serology.

The use of the FPA for the diagnosis of brucellosis (Nielsen and Gall, 2001) presents many advantages over conventional tests and compared to the AlphaLISA it has shorter incubation times and requires no serum predilution. However, it does require two separate measurements of the sample, one pre and post antigen addition. A recent validation study demonstrated that the DSp and DSn of the FPA are significantly inferior to that of iELISA for the diagnosis of *B. melitensis* infection in sheep and goats (Minas et al., 2007). The same study also demonstrated that changes of ambient temperatures within the range of 19 to 27 °C significantly affect the test result. More generally, the FPA method is restricted in its application to antigens of relatively small size owing to the short lifetime of the fluorescein label. The AlphaLISA technology has much greater flexibility and can work effectively with large molecules and immune complexes as the beads need only be within the relatively large distance of 200 nm from each other for the fluorescent reaction to progress (Von Leoprechting et al., 2004).

In conclusion, the *Brucella* AlphaLISA test presented here demonstrates diagnostic properties similar to those of more established methods, including its parent test the cELISA, and meets the standards already established for the ELISA tests by the OIE and the European Union. Its main advantage over the ELISA methods is its homogeneous nature and the inherent advantages in efficiency, precision and quality that this brings. It is a microtitre based test and can be performed in low volume, with good reproducibility and low hands-on time, making it well suited to high throughput testing. These properties combined with the relative ease with which existing diagnostic reactions, especially those in existing cELISA format, can be converted to AlphaLISA makes the technology an attractive option in the choice of platform for future high throughput serodiagnostic requirements.

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