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Development and evaluation of diagnostic tests for the serological diagnosis of brucellosis in swine

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Summary

A competitive enzyme-linked immunosorbent assay (c-ELISA), an indirect ELISA (i-ELISA) and a dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) were developed to test for antibodies to *Brucella suis* in pig and wild boar sera. An anti-*Brucella*-LPS monoclonal antibody (MAb 4B5A) (c-ELISA and DELFIA) and an anti-swine IgG monoclonal antibody (MAb 10C2G5) (i-ELISA) were used for the three assays. The specificity (Sp) and sensitivity (Se) of the assays gave the following results: Se and Sp = 100% at a cut-off value of 61.0% (B/B_0%) for c-ELISA; Sp = 99.1% and Se = 100% at a cut-off value of 21.7% (percentage positivity: PP%) for i-ELISA; Sp = 91.0% and Se = 75% at a cut-off value of 37.0% (B/B_0%) for DELFIA. In addition, the performance of a commercial fluorescence polarisation assay (FPA), standardised for bovine sera, was evaluated in swine sera. The specificity and sensitivity obtained were both 100% at a cut-off value of 99.5 (millipolarisation unit values). These results suggest that the combination of c-ELISA, i-ELISA and FPA can be used to improve the serological diagnosis of swine brucellosis.

Keywords

Brucellosis, DELFIA, Diagnosis, Dissociation-enhanced lanthanide fluorescence immuno-assay, Enzyme-linked immunosorbent assay, ELISA, Fluorescence polarisation assay, FPA, Monoclonal antibody, Serology, Swine.

Introduction

Brucella suis is a Gram-negative, facultative intracellular bacterium that is responsible for brucellosis in humans and animals. Brucellosis in animals has a major economic impact because the infection causes abortions, stillbirths and reduces fertility in herds, while brucellosis in humans is a debilitating disease characterised by fever, sweating and pain. The mortality rate of the disease in humans is estimated at approximately 5% of cases, due to complications (16, 36). Five *B. suis* biovars have been identified to date, namely: biovars 1, 2 and 3 that are present in pigs and wild boar; European hares (*Lepus europaeus*) are also a reservoir for biovar 2. Biovar 4 mainly infects reindeer and caribou, and is not usually isolated in swine. Biovar 5 is mainly present in rodents (3, 41). A presumptive diagnosis of brucellosis can be made by microscopic examination of smears stained using the Ziehl-Neelsen method, as modified by Stamp. However, in swine, serological methods are usually considered to be the most reliable method of identifying infected animals.

Serological diagnosis of swine brucellosis is based on the same tests that have been developed for bovine brucellosis and uses competitive or indirect enzyme-linked immunosorbent assays (ELISA), anti-*Brucella* antibody agglutination tests (Rose Bengal test and buffered plate agglutination test) and the complement fixation text (CFT), although none of the tests mentioned is completely specific

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and able to detect cross-reactions with other bacteria, especially *Yersinia enterocolitica* O:9 (3, 4, 7, 34, 37, 38, 39, 40, 41). A fluorescence polarisation assay (FPA) has also been developed (3, 30). Other serological tests used in cattle can also be applied to swine. The brucellin allergic skin test is widely used to identify infected herds in some countries. A definitive diagnosis can be made by isolating *B. suis* from the infected animals. *Brucella* spp. can be isolated with a wide variety of simple culture media or by using selective media, such as the Farrell medium or modified Thayer-Martin medium. Enrichment techniques can also be used. Polymerase chain reaction (PCR) techniques are available in many laboratories (3, 41). Our study describes the development and performance evaluation of two ELISAs (c-ELISA and i-ELISA) and a dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) which uses monoclonal antibodies and, as antigen, lipopolysaccharide (LPS) derived from smooth *B. suis* colonies. This study also describes the evaluation of performance of a commercial FPA for bovines, using swine sera.

Materials and methods

Monoclonal antibodies

Anti-*Brucella* LPS monoclonal antibodies (MAbs) were produced as described previously (31). Anti-swine immunoglobulin G (IgG) MAbs were produced by intraperitoneal immunisation of Balb/c mice with 50 µg/ml purified swine IgG diluted in complete Freund's adjuvant (Sigma, St Louis, Missouri). The swine IgG had previously been purified by affinity chromatography with protein A (5, 22). Two weeks after the first immunisation, 50 µg/ml of purified IgG diluted in incomplete Freund's adjuvant (Sigma, St Louis) was inoculated. A third inoculation was performed with 50 µg/ml of swine IgG diluted in sterile phosphate-buffered saline (PBS) (0.01 M, pH 7.2). On the 46th day, an intraperitoneal booster of 50 µg/ml of IgG diluted in sterile PBS was administered. After euthanasia, the mouse splenocytes were collected and fused with murine myeloma cells of line Sp2/O-Ag-14 (American Type Culture Collection) (32). The hybridomas were cultured for two weeks in Dulbecco's modified Eagle medium containing 20% foetal bovine serum, 2 mM glutamine, 100× amphotericin-penicillin-streptomycin, 50 mg/ml gentamicin, 10 000 UI/ml nystatin and 50× HAT. The hybridomas secreting anti-swine IgG antibodies were cloned in accordance with the limit dilution method (19, 25) and screened using an i-ELISA (26) using microplates coated with purified swine IgG. The cross-reactions with swine IgM and bovine, ovine, equine and chicken IgG and IgM were also verified.

The MAb isotype was determined with Immunopure® monoclonal antibody isotyping kit I (Pierce, Rockford, Illinois), according to the instructions of the manufacturer. The MAb (IgG isotype) to be used in the ELISA was purified by affinity chromatography with protein A (5, 22) and conjugated with horse-radish peroxidase (HRP) as described in the literature (29). The MAb 4B5A to be used in the DELFIA was labelled with europium using the 'Eu-Labelling Kit' (Perkin Elmer, Waltham); the europium-labelled MAb was further purified by size-exclusion chromatography with an Hi-Load 16/60 Superdex 200 column (GE-Healthcare, Uppsala) and the labelling yield was then determined with molar ratio $Eu^{3+}/IgG_{(MAb)}$.

Preparation of *Brucella suis* lipopolysaccharide

The LPS of smooth colonies used for coating ELISA microplates was prepared by phenolic extraction of *B. suis* biovar 1 (21, 31). Briefly *B. suis*, cultured in brain heart infusion agar (BHIA), was collected by centrifugation at 4 000 g for 20 min and the pellet was resuspended in sterile deionised water; 85% phenol was added to the pellet; the mixture was pre-heated at 66°C-70°C and incubated for 20 min. The phenol extract was collected by centrifugation at 20 000 g for 20 min at 4°C. After 1:10 dilution with deionised water, the phenol extract was dialysed in a saline solution of NaCl. The LPS was precipitated overnight at –20°C with three volumes of methanol/sodium acetate; the pellet was then resuspended in

sterile deionised water and stored at –80°C. The quantity of LPS was determined by the 2-keto-3-deoxyoctonate (KDO) assay (23).

Positive and negative control sera

The positive control serum (CFT titre: 1:20; i-ELISA titre: 1:512) was obtained from pigs that had been experimentally infected with *B. suis* biovar 2. The negative control serum was obtained from healthy pigs.

Sample sera

The positive sera were obtained from 106 animals from which *B. suis* had been isolated, and the negative sera from 1 036 clinically healthy animals from brucellosis-free farms. The panel of selected sera included: 46 positive wild boar sera and 16 negative wild boar sera supplied by the *Istituto Zooprofilattico Sperimentale* in Piedmont, Liguria and Valle d'Aosta (Italy); 60 positive pig sera were supplied by the Croatian Veterinary Institute in Zagreb and 1 020 negative pig sera were obtained from brucellosis-free herds in the Abruzzo and Molise regions of Italy. All sera from the above panel were used to assess performances of indirect and competitive ELISAs, whereas a lower number of sera from the same panel were used for DELFIA and FPA.

Competitive enzyme-linked immunosorbent assay

The LPS (100 µl per well), diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6) to a final concentration of 1.6 ng/ml, was dispensed into 96-well microplates (Medium Binding Costar, Corning, New York) and incubated overnight at room temperature (RT). The plates were washed once with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 1% yeast extract (Panreac, Barcelona) in PBS-T at RT for 1 h. After three washes with PBS-T, 50 µl of PBS (MAb control), 50 µl of positive and negative control swine sera and 50 µl of each sample serum were dispensed into the wells and the microplates were incubated at RT for 1 h. The plates were then washed three times with PBS-T and incubated with 50 µl/well of anti-*Brucella*-LPS MAb HRP (MAb 4B5A) diluted 1:60 000 in PBS at RT for 1 h. After further washes, 100 µl of 3.3',5.5'-tetramethyl-benzidine solution (TMB) (Sigma, St Louis) was dispensed into each well and the plates were incubated at RT for 30 min. The reaction was stopped by adding 50 µl/well of 0.5 N sulphuric acid and the optical density (OD) was measured at 450 nm. The absorbance data obtained were normalised using the following formula:

$B/B_0\% = (OD_{450}$ sample serum/OD_{450} MAb control) × 100%.

Indirect enzyme-linked immunosorbent assay

The LPS (100 µl per well), diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6) to a final concentration of 4 ng/ml, was dispensed into 96-well microplates (Medium Binding Costar, Corning, New York) and incubated overnight. The plates were washed once with PBS-T and blocked with 1% yeast extract in PBS-T at RT for 1 h. After three washes with PBS-T, 50 µl of PBS (MAb control), 50 µl of positive and negative control swine sera and 50 µl of each sample serum were dispensed into the wells and the microplates were incubated at RT for 1 h. The plates were then washed three times with PBS-T and incubated with 50 µl/well of anti-swine IgG MAb-HRP (MAb 10C2G5) diluted 1:15 000 in PBS at RT for 1 h. After further washes, 100 µl of TMB was dispensed into each well and the plates were incubated at RT for 30 min. The reaction was stopped by adding 50 µl/well of 0.5 N sulphuric acid and the OD was measured at 450 nm. The absorbance data obtained were normalised using the following formula:

Percentage positivity (PP%) = [(OD_{450} sample serum – OD_{450} negative control)/(OD_{450} positive control – OD_{450} negative control)] × 100%.

Dissociation-enhanced lanthanide fluorescence immunoassay

The LPS (100 µl per well), diluted in 0.1 M sodium phosphate buffer (pH 7.4) to a final concentration of 3.4 ng/ml, was dispensed into 96-well microplates (Polysorp-clear FluoroNunc/LumiNunc, Nunc, Roskilde) and incubated overnight at RT. The plates were washed once with DELFIA washing buffer (Perkin Elmer-Wallac, Turku) and blocked with 0.5% bovine

serum albumin in 0.05 M Tris-HCl (pH 7.4) at RT for 2 h. After three washes, 50 µl of DELFIA dilution buffer (Perkin Elmer-Wallac, Turku) (MAb control), 50 µl of positive and negative control swine sera and 50 µl of each sample serum were dispensed into the wells, and the plates were incubated a RT for 1 h. The plates were then washed three times and incubated with 50 µl/well of MAb-Eu anti-*Brucella*-LPS (MAb 4B5A) diluted 1:20 000 in DELFIA dilution buffer at RT for 1 h. After six washes, 200 µl of enhancement solution (Perkin Elmer-Wallac, Turku) was added to each well and the plates were incubated at RT for 5 min after gentle stirring. The fluorescence of the europium, recorded in counts per second (cps), was measured with the Victor ^{3}V 1420 Multilabel Counter (Perkin Elmer-Wallac, Turku).

The fluorescence data obtained were normalised using the following formula:
$B/B_0\% = (\text{cps sample serum}/\text{cps MAb control}) \times 100\%$.

Fluorescence polarisation assay

The FPA was performed with the *Brucella abortus* antibody test kit (Diachemix, Milwaukee, Wisconsin). A total of 180 µl of buffer for the FPA was dispensed into the wells of black 96-well microplates (Greiner-Bio-One, Frickenhausen); 20 µl/well of positive and negative control sera and of each sample serum was then added. The microplates were incubated for 3 min at RT and the natural fluorescence of the sera was measured with a Genios polarimeter (Tecan, Männedorf) calibrated with 1 nM fluorescein (excitation filter 485 nm; emission filter 535 nm; number of flashes: 25; gain: 100). Then, 10 µl of the O polysaccharide (OPS), conjugated with fluorescein, was dispensed into each well and the plates were incubated for 2 min at RT with gentle stirring. The fluorescence of the OPS antigen in each well was measured again with the polarimeter and the fluorescence values thus obtained were corrected by subtracting the natural fluorescence value of the sera in the corresponding wells.

The values obtained were converted to millipolarisation units (mP) using the following formula:
$mP = [(Iv-Ih)/(Iv+Ih)] \times 1\,000$
where: Iv= intensity of parallel light, and
Ih = intensity of perpendicular light.

Determination of cut-off values

The optimum cut-off values for c-ELISA and i-ELISA were determined with receiver operative curves (ROC curves) (17, 33) using the OD_{450} values of the sera analysed, normalised in relation to the OD_{450} of the MAb control ($B/B_0\%$) and the OD_{450} of the positive and negative control (PP%), respectively.

The cut-off value of the DELFIA was determined by analysing 30 swine sera which tested negative for antibodies to *B. suis*. The cut-off value was calculated by subtracting three standard deviations from the mean value of the normalised data ($B/B_0\%$) deriving from an analysis of the group of negative sera.

The optimum cut-off value of the FPA was determined with ROC curves using the fluorescence values (mP) of the sera analysed.

Results

Monoclonal antibodies

To develop the c-ELISA, we used the anti-*Brucella*-LPS MAb 4B5A, previously described to develop a c-ELISA for the detection of *Brucella* spp. antibodies in cattle, sheep and goat sera (31). The cross-reactions of MAb 4B5A are shown in Table I. MAb cross-reacts with *B. suis* biovar 1 that was used in this study for microplate coating.

Six MAbs specific for swine IgG were obtained, all of which belonged to class IgG_1, kappa light chain. The cross-reactions of anti-swine IgG MAb are shown in Table II; MAb antibodies with an OD_{450} greater than 0.3 were considered to be cross-reactive. MAb 10C2G5 was selected because it discriminated better between positive and negative sera in the i-ELISA for anti-*B. suis* antibodies.

Table I
Cross-reactions of anti-lipoploysaccharide-*Brucella* monoclonal antibody 4B5A in indirect enzyme-linked immunosorbent assay

Bacterial strain	Monoclonal antibody 4B5A*
Brucella melitensis biovar 2	100.0
Brucella melitensis biovar 1	89.0
Brucella melitensis biovar 1 Rev.1	88.9
Brucella abortus strain S19	104.0
Brucella abortus strain S99	98.0
Brucella abortus strain S99 (lipoploysaccharide)	99.8
Brucella abortus biovar 2	100.0
Brucella abortus biovar 3	102.0
Brucella abortus biovar 6	87.0
Brucella suis biovar 1	92.0
Brucella ovis	1.0
Salmonella Enteritidis	0.0
Salmonella Typhimurium	1.0
Escherichia coli O157:H7	0.0
Yersinia enterocolitica O:9	0.0
Yersinia enterocolitica O:8	0.0
Vibrio cholerae	3.0
Listeria monocytogenes	7.0
Campylobacter jejuni	7.7
Pseudomonas maltophilia	1.1

* (OD_{450} ×/OD_{450} *B. melitensis* biovar 2) × 100

Table II
Cross-reactions of anti-swine IgG monoclonal antibodies in indirect enzyme-linked immunosorbent assay (OD_{450})

MAbs	Antigen									
	Swine IgG	Swine IgM	Equine IgG	Equine IgM	Ovine IgG	Ovine IgM	Bovine IgG	Bovine IgM	Chicken IgG	Chicken IgM
8E10C9	3.4	3.4	Neg	Neg	Neg	Neg	Neg	Neg	1.5	1.2
8E10D9	1.6	1.5	Neg	Neg	Neg	Neg	Neg	Neg	1.5	1.5
8E10E8	3.4	3.4	Neg	Neg	Neg	Neg	Neg	Neg	1.2	1.3
8E10F9	1.5	1.7	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
10C2G5	>4	2.9	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
10C2C11	3.5	2.5	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Mabs monoclonal antibodies
Ig immunoglobulin
Neg negative

MAb 4B5A and MAb 10C2G5 were both conjugated with HRP to be used in c-ELISA and i-ELISA respectively; MAb 4B5A was also conjugated with europium for use in the DELFIA.

Competitive enzyme-linked immunosorbent assay

A total of 80 positive and 1 036 negative sera from the available panel were tested using the c-ELISA; the distribution of the B/B_0% values is shown in Figure 1. The B/B_0% value of 61.0% was selected as the cut-off value for the test

(Fig. 2). Sera were classified as positive if their B/B0% value was less than 61.0% and negative if their B/B0% value was greater than or equal to 61.0%. All the positive sera tested positive in c-ELISA and all the negative sera tested negative; the specificity and sensitivity of the assay were both 100%, as shown in Table III.

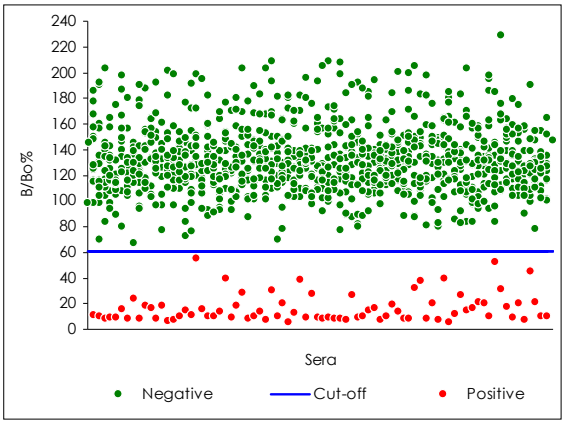

Figure 1
Competitive enzyme-linked immunosorbent assay: distribution of B/B0% values of 80 positive and 1 036 negative sera
The horizontal line represents the cut-off value (B/B0% = 61.0%)

Indirect enzyme-linked immunosorbent assay

For the i-ELISA, 106 positive and 982 negative sera from the available panel were analysed; the distribution of the PP% values is shown in Figure 3. The PP% value of 21.7% was chosen as the cut-off (Fig. 4) to obtain maximum sensitivity. The sera were classified as positive if their PP% value was greater than or equal to

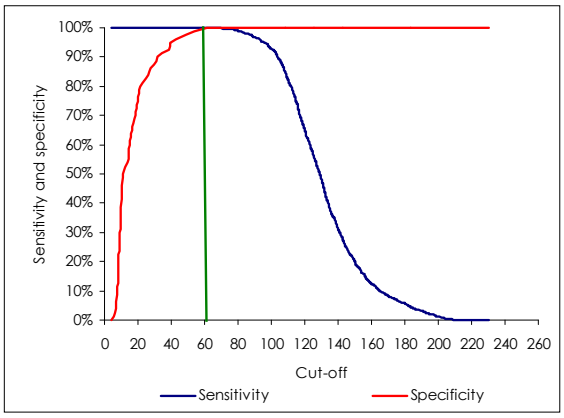

Figure 2
Cut-off value of competitive enzyme-linked immunosorbent assay calculated according to sensitivity and specificity values

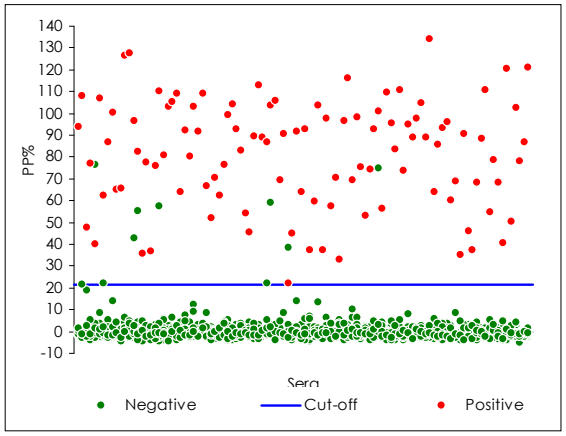

Figure 3
Indirect enzyme-linked immunosorbent assay: distribution of PP% values of 106 positive and 982 negative sera
The horizontal line represents the cut-off value (percentage positivity [PP%] = 21.7%)

Table III
Comparison between competitive enzyme-linked immunosorbent assay and isolation of *Brucella suis*; sensitivity and specificity of c-ELISA

Competitive enzyme-linked immunosorbent assay	Isolation of *Brucella suis*		
	Positive	Negative	Total
Positive	80	0	80
Negative	0	1 036	1 036
Total	80	1 036	1 116
Percentage	Lower confidence limit*		Upper confidence limit*
Sensitivity 100.0	96.4		100.0
Specificity 100.0	99.7		100.0

* 95% confidence interval

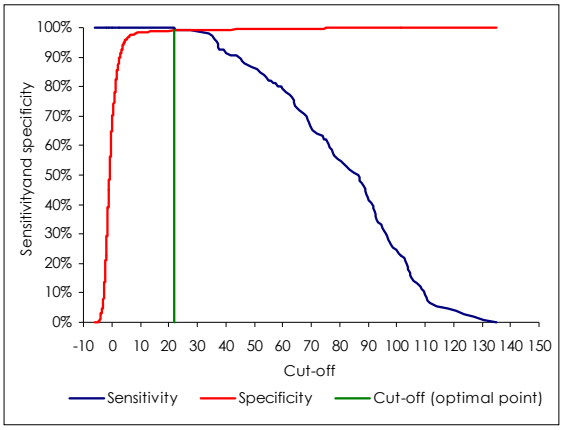

Figure 4
Cut-off value of indirect enzyme-linked immunosorbent assay calculated according to sensitivity and specificity values

21.7% and negative if their PP% value was less than 21.7%. All positive sera tested positive in i-ELISA; 9 negative sera proved to be false-positives and the remaining 973 negative sera tested negative; the specificity and sensitivity of the test were 99.1% and 100.0% respectively, as shown in Table IV.

Dissociation-enhanced lanthanide fluorescence immunoassay

DELFIA was tested using 60 positive and 212 negative sera from the same panel; the distribution of the B/B_0% values is shown in Figures 5 and 6. The B/B_0% value of 37% was calculated as the cut-off (mean of the B/B_0% values of the 30 negative sera tested – 3 standard deviations): the sera were classified as positive if their B/B_0% value was less than 37% and negative if their B/B_0% value was greater than or equal to 37%. A total of 20 of the 212 negative sera proved to be false-positives, and 15 of the 60 positive sera proved to be false-negatives; the specificity and sensitivity of the test were 91% and 75%, respectively, as shown in Table V. Due to the low performances of the test with either false-positive and false-negative results, no additional samples were analysed with this method. The labelling yield of MAb 4B5A with europium was 8.2 (expected yield value of labelling for monoclonal antibodies: 6-10).

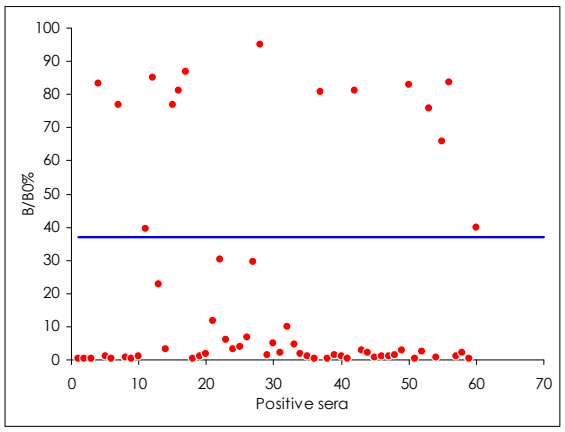

Figure 5
Dissociation-enhanced lanthanide fluorescence immunoassay: distribution of B/B_0% values of 60 positive sera
The horizontal line represents the cut-off value (B/B_0% = 37%)

Table IV
Comparison between indirect enzyme-linked immunosorbent assay and isolation of *Brucella suis*; sensitivity and specificity of i-ELISA

Indirect enzyme-linked immunosorbent assay	Isolation of *Brucella suis*		
	Positive	Negative	Total
Positive	106	9	115
Negative	0	973	973
Total	106	982	1 088
Percentage	Lower confidence limit*		Upper confidence limit*
Sensitivity 100.0	97.2		100.0
Specificity 99.1	98.3		99.5

* 95% confidence interval

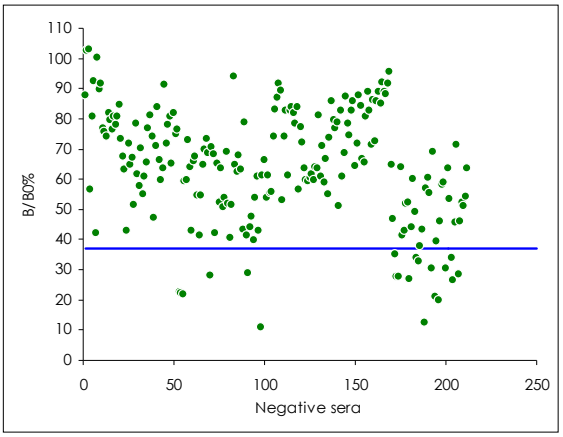

Figure 6
Dissociation-enhanced lanthanide fluorescence immunoassay: distribution of B/B0% values of 212 negative sera
The horizontal line represents the cut-off value (B/B0% = 37%)

Fluorescence polarisation assay

The commercial FPA is presently validated for bovine sera alone. To confirm the validity of the assay in swine, we tested 77 positive and 750 negative sera from the selected panel; the distribution of the mP values is shown in Figure 7. The mP value of 99.5 was chosen as the cut-off (Fig. 8) and the sera were classified as positive if their mP value exceeded 99.5 and negative if their mP value was less than or equal to 99.5. All the positive sera tested positive in the FPA and all the negative sera tested negative; the specificity and sensitivity of the assay were both 100%, as shown in Table VI.

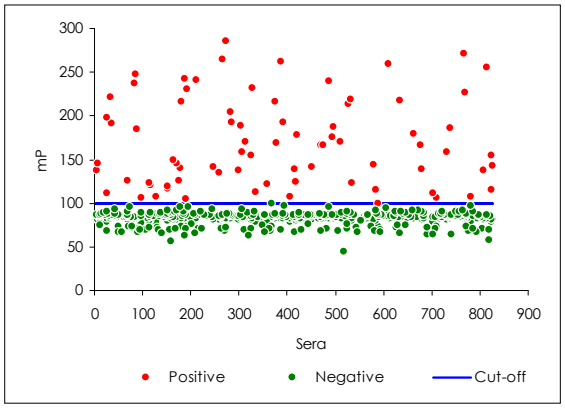

Figure 7
Fluorescence polarisation assay: distribution of millipolarisation (mP) unit values of 77 positive and 750 negative sera
The horizontal line represents the cut-off value (mP = 99.5)

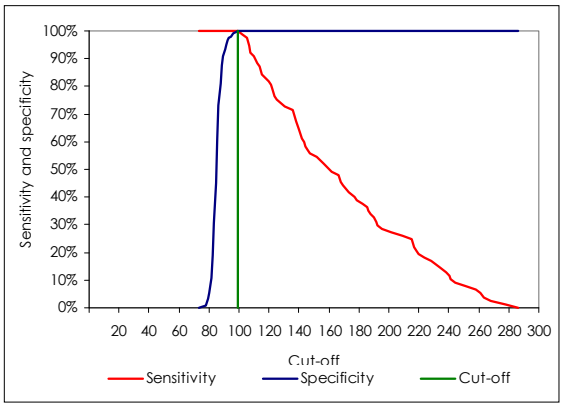

Figure 8
Cut-off values of fluorescence polarisation assay calculated according to sensitivity and specificity values

Table V
Comparison between dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) and isolation of *Brucella suis* and sensitivity and specificity of DELFIA

Dissociation-enhanced lanthanide fluorescence immunoassay	Isolation of *Brucella suis*		
	Positive	Negative	Total
Positive	45	20	65
Negative	15	192	207
Total	60	212	272
Percentage	Lower confidence limit*		Upper confidence limit*
Sensitivity 75.0	–		–
Specificity 91.0	–		–

* 95% confidence interval

Table VI
Comparison between fluorescence polarisation assay (FPA) and isolation of *Brucella suis*; sensitivity and specificity of FPA

Fluorescence polarisation assay	Isolation of *Brucella suis*		
	Positive	Negative	Total
Positive	77	0	77
Negative	0	750	750
Total	77	750	827
Percentage	Lower confidence limit *		Upper confidence limit*
Sensitivity 100.0	96.2		100.0
Specificity 100.0	99.2		100.0

* 95% confidence interval

Discussion

Swine brucellosis is mainly caused by *B. suis* biovars 1, 2 and 3; the other species of *Brucella* occasionally reported in swine are *B. abortus* and *B. melitensis* (3). *B. suis* biovar 1 is common in South America and Asia, whilst *B. suis* biovars 1 and 3 have been reported in the United States, in Australia and in the People's Republic of China. *B. suis* biovar 2, the natural reservoir of which is the wild boar and/or the European hare, is the most common strain isolated in Europe (1, 4, 9, 11, 15, 24, 28), but biovar 1 and 3 are also present. In Italy, the isolation of *B. suis* biovars 1 and 2 has been reported (8, 10, 12, 18). *B. suis* biovar 2 is rarely pathogenic in humans, whereas *B. suis* biovars 1 and 3 are highly pathogenic and can cause serious illness (3, 4, 41).

Swine brucellosis was eradicated from many European countries in the 1970s, as a result of the industrialisation of farms. In the 1990s, the disease re-emerged in extensive husbandry, transmitted by hares and wild boars. The available serological tests for swine brucellosis described in the World Organisation for Animal Health (*Office International des Épizooties*: OIE) *Manual of diagnostic tests and vaccines* (41), are the i-ELISA and c-ELISA, the Rose Bengal rapid agglutination test (RBT), the FPA and, although not recommended, the CFT. None of the above-mentioned tests has optimal sensitivity values, and even less so those for specificity for individual diagnostic purposes due to the false-positive reactions which probably arise from cross-reactions with other bacteria and mainly with *Y. enterocolitica* O:9. Swine serum may sometimes contain non-specific antibodies, probably IgM, that reduce the specificity of conventional tests, especially for serum agglutination tests. Moreover, swine complement interacts with guinea-pig complement to produce pro-complementary activity that reduces the sensitivity of the CFT (41).

Conclusions

The aim of this study was to evaluate the methods described in the international literature for the diagnosis of swine brucellosis and to develop new and more specific methods. For this purpose, we assessed the diagnostic sensitivity and specificity of i-ELISA and c-ELISA methods and of the DELFIA, developed with *B. suis* LPS (homologous antigen) on swine sera. In addition, the performance of a commercial FPA was also evaluated in swine.

With the c-ELISA, the maximum sensitivity (confidence interval between 96.4% and 100.0%) and the maximum specificity (confidence interval between 99.7% and 100.0%) were obtained at a cut-off value of 61.0% (B/B0%). With the i-ELISA, the maximum sensitivity (confidence interval between 97.2% and 100.0%) and the maximum specificity (confidence interval between 98.3% and 99.5%) were obtained when the cut-off value was set at 21.7% (PP%). The FPA, which showed high levels of specificity (confidence interval between 99.2% and 100.0%) and of

sensitivity (confidence interval between 96.2% and 100.0%) can be used as a screening method if performances recorded in this study are be confirmed by field tests.

The DELFIA method, which is already in use to test for steroid hormones and veterinary drug residues, cytokines, antibodies and bacterial and viral antigens (2, 6, 13, 14, 20, 27, 35), does not offer good levels of specificity and sensitivity at this stage of development (91% and 75%, respectively) and it is not expected to be used widely in routine diagnosis in the view of the high cost of the europium used in the method.

Our results suggest that a combination of the c-ELISA, i-ELISA and FPA methods may provide an appropriate solution for the serological diagnosis of swine brucellosis due to their high sensitivity and specificity. However the lack of an international standard serum, such as that for cattle, sheep and goat brucellosis, still represent the major limitation for the standardisation of diagnostic tests for serological diagnosis of swine brucellosis.

Finally a pilot study for the field application of a diagnostic protocol, involving the combined use of the three tests described above with a defined cut-off, is required to evaluate real performances of the assays in field conditions and, in particular, to evaluate sensitivity during outbreaks and specificity to avoid false-positive reactions in disease-free herds.

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