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Evaluation of Competitive ELISA for Detection of Antibodies to *Brucella* Infection in Domestic Animals

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Aim To evaluate competitive enzyme-linked immunosorbent assay (cELISA) for its suitability as an additional serological test for the diagnosis of animal brucellosis.

Methods cELISA, which was developed at the Veterinary Laboratories Agency, has been evaluated for its accuracy and suitability as an additional serological test for the diagnosis of animal brucellosis. Samples from naturally and experimentally infected animals and those from *Brucella*-free flocks and herds were tested.

Results Data obtained since 1991 were analyzed from routine surveillance, animals experimentally infected with *Brucella*, and stored sera to validate cELISA for the detection of antibodies to *Brucella* in cows, small ruminants, and pigs. The sensitivity of the test ranged from 92.31% to 100%, in comparison with 77.14% to 100% for the complement fixation test (CFT). Specificities for cELISA, indirect enzyme-linked immunosorbent assay, and CFT were greater than 90%.

Conclusion cELISA can be used on a variety of animal species, and an added advantage is its suitability for use on poor-quality samples such as those affected by hemolysis.

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In accordance with EC Directive 91/68/EEC, flocks of sheep and herds of goats in the United Kingdom (UK)are monitored serologically to prove that they are free from Brucella melitensis. In 2006, competitive enzyme-linked immunosorbent assay (cELISA) was introduced to screen these animals as part of a surveillance program in Great Britain (GB), the territory including all of the UK except for Northern Ireland. It replaced the complement fixation test (CFT) because of its much higher specificity and ease of automation. Currently, in excess of 35 000 animals are tested annually.

In 2001, a revision to the pig semen directive was introduced by EC Directive 99/608 so that CFT was replaced with the Rose Bengal test (RBT) as the test used for brucellosis on all pigs whose semen is used for artificial insemination. RBT and CFT were run in parallel in addition to cELISA prior to this date in order to assess the effects of changing the testing regime and, at the same time, to validate the use of cELISA for pigs. During 2001, all routine samples that were tested for artificial insemination purposes and were positive by RBT were also tested by cELISA and the results analyzed using different diagnostic thresholds. The aim was to set an appropriate threshold that would provide optimal specificity and sensitivity for cELISA.

CFT, RBT, and indirect enzyme linked immunosorbent assay (iELISA) are the conventionally used tests for diagnosis of bovine brucellosis. These tests are described in the Manual for Diagnostic Tests and Vaccines for Terrestrial Animals produced by the World Organisation for Animal Health, previously the Office International des Epizooties (OIE) (1), and this manual gives details of all the diagnostic methods. It also describes the strain of *Brucella* required for antigen preparation and the procedure for standardization for each test.

The cELISA for the detection of antibodies against *Brucella* spp. was adapted at the Veterinary Laboratories Agency (VLA) from the method described by MacMillan et al in 1990 (2). It was initially developed for the diagnosis of brucellosis in small ruminants and was tested extensively on British sheep and on sheep and goats from France. It has also since been tested on large numbers of cattle and pigs.

The aim of this study was to bring together and compare all brucellosis testing results carried out using cELISA, RBT, and iELISA at the VLA since 1991. The samples had been collected and analyzed within the framework of various surveillance screening programs and experimental studies. The present study demonstrates the effectiveness of cELISA compared with other assays currently used as diagnostic tests of brucellosis in domestic animals.

METHODS

Study design

This study performed retrospective comparisons of diagnostic testing of samples that had been collected at different periods from various regions of France and GB. For example, approximately 1 million bovine blood samples were routinely tested annually for evidence of *Brucella* infection up until 2007. Samples had been collected and analyzed in previous years as described below. In some cases, samples previously collected and stored frozen were thawed and tested for the purposes of this study. A proportion of samples were from animals that were found to be unsuitable for testing by confirmatory tests, most often CFT. cELISA was used on all samples unsuitable for CFT as it is technically straightforward to perform and is unaffected by the age and condition of the sample (3).

All animal work was approved by the VLA ethics committee and is in line with the Animal (Scientific Procedures) Act.

Optimization of antigen and monoclonal antibody for cELISA

An M dominant epitope lipopolysaccharide was extracted from *Brucella melitensis* strain 16M by the hot phenol method (4) as the antigen for coating the plates. An anti-M epitope monoclonal antibody (mAb) BM40 (5) was conjugated with horseradish peroxidase (HRP) by the method adapted from Nakane and Kawaoi (6). HRP (20 mg) was dissolved in 5 mL of distilled water, and 1 mL of freshly prepared 0.1 M sodium periodate was added. After stirring for 20 minutes at room temperature, the activated HRP was dialyzed against 1 mM sodium acetate (pH 4.0) for 15 to 20 hours. The dialyzed HRP and 20 mg of mAb were separately adjusted to pH 9 with 10 mM sodium carbonate buffer (pH 9.5) before being combined and then stirred at room temperature for 2 hours. Ascorbic acid (4 mg/mL, 0.5 mL) was added and after 4 hours of incubation at 4°C with continuous stirring, the mixture was dialyzed between 15 and 20 hours against several changes of 0.1 M phosphate-buffered saline. The conjugate was filtered through a 100-kDa microcentrifuge filter (Sigma M-2286), added to

an equal volume of glycerol and stored at -20°C until required.

The optimal concentration of antigen and conjugate for use in cELISA was determined by titration against standard sera from a known brucellosis-free sheep and serum from a goat experimentally infected with *B. melitensis* strain H38. The M dominant *B. melitensis* antigen and anti-M mAb were selected because the M epitope is considered to be specific to brucellosis, which may help to reduce the incidence of false positive reactions caused by antibodies produced in response to infection by other bacteria. Antibodies produced as a result of infection with all *Brucella* smooth strains may compete with BM40 (7) for epitopes on the lipopolysaccharide O-chain (8).

Conventional and cELISA diagnostic testing

Subjective tests (CFT, RBT) were carried out by trained technicians using standard techniques (1). The cELISA as adapted by MacMillan (2) was carried out according to Stack et al (3). Two thresholds have been used during the course of this work. From 1991-1994, it was initially set so that samples whose optical densities at 450 nm were equal to or less than 75% of the negative control serum were considered positive. In 1994, the threshold for positivity was changed to be optical densities equal to or less than 60% of the conjugate control. This change was made to more accurately measure the inhibition of conjugated mAb binding.

Cattle

Non-infected animals. Samples from the 1994 domestic surveillance program were tested by cELISA. The samples comprised 640 animals that were negative by CFT, defined as less than 20 CFT International Units per mL, and 160 negative by iELISA. In 2004, from a total 835278 animals in the domestic survey program in GB, 5608 animals that were classified as false positives by iELISA were tested by cELISA. In 2006, a further 2000 animals from GB were randomly selected from the surveillance program and tested by cELISA and iELISA.

Infected animals. A total of 147 serum samples from individual cattle confirmed by culture as infected by *B. abortus* biovar 1 had been collected in GB during the 1970s and early 1980s when brucellosis was still endemic. The sera had been kept at less than -20°C until their use in the present study. They were then tested by cELISA and CFT.

Sheep and goats

Non-infected animals. Samples from 462 sheep and 2000 goat samples from a previous research project carried out in 1991 were used. They had been collected from brucellosis-free flocks and herds in GB that were found to be negative during RBT screening. In 1992, iELISA and cELISA were used to test a further 6260 samples from sheep and goats from GB and 433 goats and 309 sheep from France. From the annual surveillance program for diagnosis of *B. melitensis* in sheep and goats in 2006, another 36785 brucellosis-free animals were tested using cELISA alone.

Infected animals. CFT and cELISA results were used from 30 goats and 35 sheep from France that had been naturally infected with *Brucella melitensis*. Both tests were also used to analyze additional 23 goats from France and 22 sheep from GB that had been experimentally infected with *B. melitensis* strain H38 and bled at regular times post infection.

Pigs

Non-infected animals. From the routine samples collected during 2001 from pigs in GB, 2031 samples were tested using RBT and cELISA. In 2002, a further 1488 animals from herds in GB were tested by cELISA, RBT, and CFT.

Infected animals. Samples were obtained from 13 animals that tested culture-positive for *B. suis:* 10 came from the US National Veterinary Services Laboratories and 3 from the State Veterinary Service in Denmark. These samples were tested by CFT and cELISA. In addition, 588 samples obtained from herds from France where at least 1 animal was culture-positive were tested by RBT, CFT, and cELISA.

Positive controls for all species. For cELISA and iELISA the positive control was from a goat artificially infected with *B. melitensis* H38. The positive controls used for RBT, CFT, and iELISA were bovine and of known titer, based on the OIE *B. abortus* International Reference Standard Serum.

Negative controls for all species. A pool of species-specific sera from *Brucella*-free animals in GB was used for all assays; these sera had originally been screened by RBT, iELISA, or cELISA.

No vaccinated animals were included in this study due to the insufficient number of samples with reliable provenance.

RESULTS

The data obtained from the non-infected animals is shown in Table 1 and from infected animals in Table 2.

Sheep and goats

The specificity for sheep and goats, as seen in Table 1, is comparable for both cELISA and iELISA, with greater than 99% specificity for both tests. In 2006, the first full year when surveillance was carried out using cELISA instead of CFT, 36 785 animals were tested with only one false positive, giving greater than 99.99% specificity (Table 1).

The data from the naturally infected flocks and herds (Table 2) shows that the sensitivity of cELISA (94.29%) is better than that of CFT (77.14%) for sheep but is the same for goats (96.67%).

From the experimentally infected sheep in which samples were taken at regular time points post-infection, all samples (Table 2) were positive by cELISA and CFT, but cELISA detected infection in 12 of the 22 (55%) sheep 2-23 days earlier than CFT. For 6 (27%) of these animals the difference

TABLE 1. Results of testing of non infected animals randomly selected from herds in Great Britain and France*

			iELISA		cELISA	
Animals	Source	Total No. of samples	negative samples	specificity	negative samples	specificity
Sheep	United Kingdom	462	460	99.57	461	99.78
Sheep	France	309	307	99.35	308	99.68
Goats	United Kingdom	2000	1991	99.55	1999	99.95
Goats	France	433	433	100	432	99.77
Sheep and goats	Great Britain	6260	6250	99.84	6258	99.97
Sheep and goats	Great Britain	36 785	ND	NA	36 784	99.99
Cattle	Great Britain	160	160	NA	159	99.38
Cattle	Great Britain	5608	0	NA	5605	99.95
Cattle	Great Britain	2000	2000	100	1999	99.95
			RBT-negative samples	RBT specificity		
Pig	Great Britain	2031	1777	87.49	2004	98.67
Pig	Great Britain	1488	1269	85.28	1464	98.38
			CFT-negative samples	CFT specificity		
Cattle	Great Britain	640	640	NA	640	100
Pig	Great Britain	1488	1389	93.34	1464	98.38

*ND – not done; NA – not applicable; iELISA – indirect enzyme-linked immunosorbent assay; cELISA – competitive ELISA; RBT – Rose Bengal test; CFT – complement fixation test.

TABLE 2. Results of testing of naturally and experimentally infected animals

Species	Source	Total No. of samples	Complement fixation test-positive samples	Sensitivity	Competitive enzyme-linked immunosorbent assay-positive samples	Sensitivity
Sheep*	United Kingdom	22	22	100	22	100
Sheep†	France	35	27	77.14	33	94.29
Goats*	United Kingdom	23	23	100	23	100
Goats†	France	30	29	96.67	29	96.67
Cattle†	United Kingdom	147	137	93.2	144	97.96
Pig*†	United States	10	8	80	9	90
Pig†	Denmark	3	3	100	3	100
Pig‡	France	588	260	44.22	312	53.06

*Experimentally infected.
†Naturally infected.
‡Herd where at least one culture-positive animal was known.

was greater than 6 days. Among the 23 goats that were bled at regular time points post-infection, 15 animals (65%) were detected positive 14-18 days earlier by cELISA than by CFT. No animals were detected earlier by CFT.

Cattle

The specificity of cELISA was comparable to that of CFT and iELISA for those samples that were originally negative by either iELISA or CFT. Of the 5608 false iELISA positives from the surveillance program, only 3 (0.05%) were positive by cELISA (Table 1).

Table 2 shows that the sensitivity of cELISA is better than that of CFT. Samples from culture-positive animals totaled 147, 144 were cELISA-positive (97.96% sensitivity), and 139 were CFT-positive (94.56% sensitivity).

Pigs

From the routine samples collected from pigs in GB and screened by RBT in 2001, 254 of the 2031 were RBT false positives, giving 87.49% specificity. With the threshold set at 60% of the mean optical density of the conjugate control (as for cattle, sheep, and goats at this time), only 27 were false positives by cELISA, corresponding to 98.67% specificity. The number of false positives rose to 37 if the threshold was set at 75%, corresponding to 98.17% specificity. Of the additional 1488 animals tested, the number of false positives and specificity for each method were as follows: RBT, 219 false positives and 85.28% specificity; cELISA, 24 and 98.38%; CFT, 99 and 93.34%. Additional 56 animals were anticomplementary and therefore required re-sampling (Table 1).

Twelve of the 13 culture-positive animals were positive by both cELISA and CFT. From the 588 animals for which the herd contained at least one culture-positive animal, 312 (53%) were positive by cELISA, 260 (44%) by CFT, and 434 (74%) by RBT (Table 2).

DISCUSSION

cELISA showed a higher specificity than either RBT or CFT for pig samples, but for other domestic animals it was only marginally more specific than iELISA. Generally more animals from the infected herds were positive by cELISA than by CFT. The exception to this is in pigs, in which there were fewer positives by cELISA than RBT. Some of the "additional" positive pigs detected by RBT may be attributed to false positive reactions, which are notorious in pigs (9). Alternatively, the data from the French pigs may be inaccurate, since the samples were obtained from herds with only one known culture-positive animal; thus, the status of the remaining animals was unknown. The high cost of being able to infect enough animals for experimental infection and the difficulty of obtaining material from known infected animals make conclusive determination of sensitivity difficult. More studies need to be done on infected pigs to elucidate sensitivity and determine a suitable threshold for positivity.

The use of cELISA in addition to, or as a replacement for, conventional tests for brucellosis offers many benefits. Samples that are received in the laboratory that have deteriorated are often un-testable. These samples delay results and can incur additional costs to the farmer if animals have to be re-bled. An additional validated test is a necessity in these situations if not to entirely replace assays currently used, then to provide extra information. This applies to most domestic species but from our experience at VLA, porcine samples are the ones that most frequently suffer hemolysis or anticomplementary reactions.

The cELISA procedures for detection of porcine antibody to *Brucella* spp. are identical to those used for bovine antibody to *B. abortus* and *B. melitensis,* making the test valid for multiple animal species. This assay is capable of eliminating some reactions due possibly to *Y. enterocolitica* serotype 0.9 or other cross-reacting antibodies, such as IgM, which have lower affinity for *Brucella* epitopes than does the mAb used in the assay (10). cELISA is a prescribed test for international trade but none of the conventional serological tests has been shown to be entirely reliable for routine diagnosis in individual pigs (1).

There is continuing validation from the annual surveillance program for *B. melitensis* carried out in GB, providing results for more than 35 000 sheep and goats a year. Since it replaced CFT, cELISA has reduced the number of false positives and the number of un-testable samples. cELISA is also being used to test serum and body fluids from marine mammals (unpublished data), since it is not animal species-specific and can be used on poor-quality samples where some conventional tests are unsuitable. Often material from marine mammals is of poor quality because it has been obtained from carcasses that may have been dead for some time.

cELISA is a rapid assay, it is faster than CFT, and it can be automated and therefore the results can be measured objec-

tively. This assay incorporates an mAb similar to that used by Stack et al (3), McGiven et al (11), and Jungersen et al (9) but different from that used in other reports (12-14), so it adds to the information we already have from the battery of tests currently used for brucellosis diagnosis. Continuing validation is ongoing as samples with reliable provenance become available.

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