The first International Standard anti-*Brucella melitensis* Serum

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Summary

The World Organisation for Animal Health (OIE) requested an International Standard anti-*Brucella melitensis* Serum (ISaBmS) to standardise diagnostic tests and reagents for sheep and goats. The agreed criteria were the highest dilution (in negative serum) of the standard which must give a positive result and the lowest dilution (in negative serum) which must simultaneously give a negative result. The two dilutions for each assay were, respectively: indirect enzyme-linked immunosorbent assay (iELISA) 1/64 and 1/750, competitive ELISA (cELISA) 1/8 and 1/300, fluorescent polarisation assay (FPA) 1/16 and 1/200, Rose Bengal test (RBT) 1/16 and 1/200. The OIE International Standard Serum (OIEISS) will remain the primary standard for the RBT; the ISaBmS is an additional standard. It was impossible to set criteria for the complement fixation test, therefore the OIEISS will remain the primary standard. The ISaBmS can be used to standardise iELISA, cELISA and FPA to diagnose sheep and goat brucellosis. This standard should facilitate harmonisation of tests used for brucellosis surveillance and international trade in these species.

Keywords

Brucella melitensis – Brucellosis – Diagnosis – Serology – Standardisation.

Introduction

Brucellosis in sheep and goats is widespread in many areas of the world, particularly in some Mediterranean and Middle Eastern countries (1, 11). *Brucella melitensis* is the main causative agent of brucellosis in sheep and goats. This disease causes a significant reduction in animal productivity and leads to restrictions on animal movements. *Brucella melitensis* is also considered one of the more virulent of the species that cause human brucellosis,

and it is frequently transmitted via ingestion of unpasteurised dairy products (8, 12). In many areas of the world more than 10% of sheep and goat flocks are infected with brucellosis; in some places the prevalence is considerably higher (1). All these factors contribute to an unacceptably high rate of human brucellosis in these areas (10). With the exception of France (5), no country has ever reliably reported that *B. melitensis* has been eradicated from small ruminants at the national level.

Control of brucellosis is usually performed by vaccination in the first instance, in order to reduce the prevalence of disease to acceptable levels (2). Prior to commencement of a vaccination strategy it is often desirable to perform serological surveys to evaluate the prevalence and spread of the disease so as to target the vaccination optimally. To eradicate the disease and to qualify for World Organisation for Animal Health (OIE) brucellosis-free or officially free status, it is necessary to conduct serosurveillance (18). The application of serological testing is also required for the international trade of animals. It is therefore important that serological tests be standardised and harmonised properly so that they provide reliable results.

The first International Standard for anti-*Brucella abortus* Serum was established in 1952 (13). In 1965, the World Health Organization requested that a second standard be prepared to replace the dwindling stocks of the first. The second OIE International Standard Serum (OIEISS) is currently available and has been applied for the standardisation and harmonisation of some of the tests currently used for the serodiagnosis of sheep and goat brucellosis associated with *B. melitensis*. These include the complement fixation test (CFT) and the Rose Bengal test (RBT) (16). This standard serum was obtained from a cow experimentally infected with *B. abortus* strain 544 (4). It is therefore not suitable for use in species-specific assays, such as the indirect enzyme-linked immunosorbent assay (iELISA), for species other than cattle. Accordingly, the need for a standard prepared from sheep or goat serum was identified. Furthermore, it was felt that a standard serum raised against infection with *B. melitensis* may be more appropriate for the standardisation of tests for sheep and goats not already covered by the OIEISS. For this reason, the OIE invited the Veterinary Laboratories Agency (VLA) in the United Kingdom to produce a standard.

It was the aim of this project to produce the first International Standard anti-*Brucella melitensis* Serum (ISaBmS), and to distribute this reagent to recognised centres of proven diagnostic excellence for full evaluation. The project was to be concluded by setting agreed minimum and maximum thresholds of analytical sensitivity using this new standard and by its distribution to National Reference Laboratories for the preparation of secondary standards.

Materials and methods

The ISaBmS was obtained by pooling seven sera from goats that had been confirmed by culture to be infected with *B. melitensis*. Four pregnant goats were experimentally infected (conjunctively with 5×10^5 colony forming units [cfu] of strain H38, biovar 1) after 19 weeks of gestation. The serum was collected from two of the goats 67 days post infection and from the other two goats 259 days post infection. One goat was experimentally infected (conjunctively with 2.9×10^7 cfu of strain H38) after 12 weeks of gestation and the serum was collected 63 days post infection. All animal procedures were conducted in accordance with the United Kingdom Animal (Scientific Procedures) Act 1986. Two serum samples came from goats naturally infected with biovar 3, which has mixed A and M antigen dominance in addition to the common 'C' epitope (15). Thus this standard should contain antibodies to both A- and M-specific epitopes of the O-polysaccharide as well as to the common epitopes (9). Pooling of sera was necessary to achieve the desired volume of material but also to obtain a sample representative of a range of humoral immune responses. This and subsequent processing was performed at the VLA, but the serum samples were obtained via donations from a number of sources, including some of the laboratories included in this analysis.

In accordance with OIE guidelines (17), all the samples contributing to the pool were free from haemolysis and excessive lipaemia. The serum was also plated on serum dextrose agar plates at 37°C in 10% CO_2 for seven days and no detectable bacterial growth was observed.

The serum standard was inactivated at 56°C for 30 min, lyophilised in 1 ml aliquots, and stored at 4°C prior to dispatch and analysis in collaborating laboratories. The contents of 36 of the glass ampoules were weighed and 13 were tested by CFT, iELISA, competitive ELISA (cELISA) and fluorescent polarisation assay (FPA) at the VLA to assess batch homogeneity prior to dispatch.

It was agreed that each of the laboratories would subject the sample to each of the appropriate routine and validated tests used in their laboratory. All of the tests that are included in the OIE *Manual for Diagnostic Tests and Vaccines for Terrestrial Animals* were performed in accordance with the methods described therein (16). Commercial kits were used according to the manufacturer's instructions.

The initial collaborative testing of the ISaBmS was undertaken in 2007 at the laboratories of each of the authors (coded randomly as A–I in the tables). Reagents and kits from the following producers (coded randomly 1–11 in the tables) were also used:

– Animal Disease Research Institute, Canada

– Centre for Research and Food Technology of Aragon (CITA), Spain

– Diachemix, United States

– Ingenasa, Spain

– Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise, Italy

– National Veterinary Research Laboratory (LNIV), Portugal

– Institut Pourquier, France

– National Service for the Quality and Safety of Agricultural Products (SENASA), Argentina

– Svanova, Sweden

– Synbiotics, France

– VLA.

Each laboratory was sent three ampoules of ISaBmS and then prepared a series of dilutions from each individual ampoule. Each laboratory was asked to make particular dilutions of the serum from each ampoule in their own well-characterised negative goat serum (directly, not by double dilution) and to test these dilutions as individual samples in the different tests. These dilutions were tested three times for each test on three separate days in order to determine which dilutions were positive, which were inconclusive and which were negative, according to the criteria of the test used.

In the case of the CFT the neat undiluted standard was tested, and the end titration result was used to determine the number of international CFT units (ICFTUs) present (based on the assay antigen being standardised against the *B. abortus* OIEISS). The list of tests used and standardised with the ISaBmS is presented in the results section. In the case of the iELISA, two more distributions and evaluations of the ISaBmS (one ampoule per laboratory) were performed using dilutions not included on the previous occasions.

All the data were sent to the VLA for collation, statistical analysis and drafting of initial conclusions. For the CFT, the quantitative data were used in a three-way nested analysis of variance (ANOVA) to identify any significant differences between laboratories, days and ampoules. A logarithmic transformation (to base 2) was first applied to the CFT results to normalise the data, to enable the appropriate application of ANOVA techniques. The quantitative data from the iELISAs were used to produce dose–response curves.

The results of this analysis were distributed to all the participants and a consensus was reached on the conclusions presented here.

Results

The average reconstituted ISaBmS ampoule content from the 36 samples was 1.039 g with a coefficient of variation (CV) of 1.12%. The results from the iELISA performed on these ampoules showed a CV of 1.73% when the samples were tested neat (in this case, however, still mixed with the test buffer according to the local protocol). The 95% confidence interval for the CV of the population iELISA results was calculated to be within the range of 1.24% to 2.89% (7). The results from the three-way nested ANOVA on the CFT data (n = 54) showed that there were no significant differences between the ampoules (p = 0.479). However, there was a significant difference when the samples were tested on different days (p = 0.004) and a highly significant difference between laboratories (p < 0.001). The average standard deviation between days within each laboratory was just 5% of a single dilution. The average difference between laboratories was less than 80% of a single dilution. The individual results for the CFT are not shown because this test will not be standardised using the ISaBmS.

The dose–response curve for the ISaBmS as determined by the iELISAs is shown in Figure 1. This figure shows the results obtained from three participating laboratories. Each graph shows the dilutions that lie on the linear section of the curve. The figure also shows some differences between laboratories in the dynamic range of this phase that reflect differences in the design of the individual assays and the assignment of positive and negative results.

The results for each of the tests used for evaluating the serum standard are shown in Tables I to V. In each table,

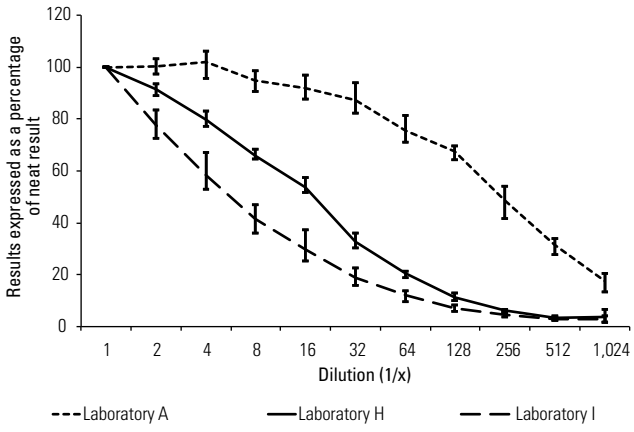

Fig. 1

Examples of the dose–response curve of the International Standard anti-*Brucella melitensis* Serum as tested in three indirect enzyme-linked immunosorbent assays (in three laboratories)

Error bars represent the maximum and minimum results from three tests, each performed on different days

the laboratory code (A to I) is shown in the first row of the table, together with a code (1 to 11) to indicate the source of the test reagents. The first column of each table indicates the dilution (in negative serum) of the ISaBmS that was tested in accordance with each of the local protocols used. The results are presented in the tables as two or three numbers (separated by '/') for each combination of laboratory and dilution. The first number indicates the number of replicates of the dilution, tested by the particular laboratory, that were classified as positive. If there are three numbers, the second indicates the number of replicates that were inconclusive (or borderline or suspicious). If there are three numbers, the third – or if there are two numbers, the second – is the number of replicates that were negative.

Not all tests have a possible inconclusive/borderline/suspicious category but if one of the methods used has one, the results of all the methods have been shown using three numbers. If none of the tests has an inconclusive/borderline/suspicious category the results are shown using just two numbers. The final column in the table shows an overall result summarised from the data in the preceding columns. The first of the two numbers in this column shows the number of laboratories that categorised the dilution as positive for the majority of replicates, and the second number shows the number of laboratories that categorised the dilution as negative for the majority of replicates.

The data in Tables I to V have also been shaded to indicate the majority categorisation of each dilution by the individual laboratories and the overall interpretation, shown as: positive (white), negative (dark grey), or inconclusive/borderline/suspicious (light grey). The shading in the first column indicates the required categorisation of each dilution that has been determined as a result of this project. Those dilutions which should be categorised as positive by the test in question are shown in white. Those that should be categorised as negative have been shaded dark grey. Those dilutions for which it is possible to have any test categorisation have been shaded light grey. These three sets of dilutions have been separated by bold lines running across the tables.

The results for the iELISA are shown in Table I. Eight laboratories submitted results for this test using a total of seven different kits. The numbers in superscript ($^{1–3}$) within the first column indicate the distribution round from which the data came. The results show that the highest dilution for which all data sets always produced positive classifications was 1/64. Although for data sets B3 and E5 this was not the case in the first distribution, it could be inferred from subsequent data (see Table I for details). It was therefore agreed that the 1/64 dilution should be the minimum analytical sensitivity threshold for the iELISA when testing the ISaBmS. The lowest dilution for which all data sets always produced entirely negative classifications was 1/750. It was therefore agreed that the maximum analytical sensitivity threshold should not exceed a 1/750 dilution of the standard.

The results for the cELISA are shown in Table II. Eight laboratories submitted results for this test using a total of five different kits. Eight out of the nine data sets categorised a 1/8 dilution as positive on all occasions. Only one data set (A8) did not detect this dilution as positive. However, two other laboratories (F and G) used the same kit and classified the 1/8 dilution of the standard as positive on every occasion. It was therefore agreed that the 1/8 dilution should be the minimum analytical sensitivity threshold for the cELISA when testing the ISaBmS. All of the data sets categorised the 1/128 dilution as negative on the majority of occasions (a total of 76 negative from 81 results), and positive results were obtained at this dilution with only one kit. At the 1/256 pre-dilution there were only two positive results out of a possible 81, again using the same kit (A7, Table II). To maintain the given safety margin it was agreed that the maximum analytical sensitivity threshold should not exceed a 1/300 dilution of the standard. No testing was performed at the 1/300 dilution but this value has been included in the table for indicative reasons, as described in the Discussion.

The results for the FPA are shown in Table III. Five laboratories submitted results for this test and all used the only available commercial kit. All laboratories categorised the 1/16 pre-dilution as positive by this test on the majority of occasions (43 out of 45). It was therefore agreed that the 1/16 dilution should be the minimum analytical sensitivity threshold for the FPA when testing the ISaBmS. All laboratories categorised the 1/128 pre-dilution as negative on the majority of occasions (40 out of 45). To maintain a given safety margin it was agreed that the maximum analytical sensitivity threshold should not exceed a 1/200 dilution of the standard. No testing was performed at the 1/200 dilution but this value has been included in the table for indicative reasons, as described in the Discussion.

The results for the RBT are shown in Table IV. Seven laboratories submitted results for this test using antigens from five different manufacturers. All tests detected the 1/16 dilution as positive and all of the tests gave a negative result for the 1/128 dilution. Accordingly it was agreed that the 1/16 dilution should be the minimum analytical sensitivity threshold for the RBT when testing the ISaBmS. To maintain a given safety margin it was agreed that the maximum analytical sensitivity threshold should not exceed a 1/200 dilution of the standard. No testing was performed at the 1/200 dilution but this value has been included in the table for indicative reasons, as described in the Discussion.

Key to Tables I to V

The test result column headings provide the laboratory code (A to I) together with the code for the source of the test reagents (1 to 11). The result columns indicate the number of positive/suspect/negative results (for the Rose Bengal test there are only two values: positive/negative). The overall column provides the number of laboratories with average results

Average result positive

Average result negative

Average result suspect (for column 1 this shading indicates that any result is acceptable)

* min-ASn: minimum analytical sensitivity requirement equal to this dilution
** <max-ASn: maximum analytical sensitivity less than this dilution

Table I
Indirect enzyme-linked immunosorbent assay results summary

Dilution	Test results										
	A2	B3	C11 (ovine kit)	C11 (caprine kit)	C9	E5	F2	G2	H7	I10	Overall
Neat [1]	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	10/0
1/2 [1]	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	10/0
1/4 [1]	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	10/0
1/8 [1]	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	10/0
1/16 [1]	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	8/1/0	9/0/0	9/0/0	9/0/0	10/0
1/32 [1]	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	8/0/1	8/1/0	9/0/0	9/0/0	9/0/0	10/0
1/50 [2]	24/0/0	4/0/0	3/0/0	3/0/0	3/0/0	3/0/0	2/0/0	9/0/0	8/0/0	6/0/0	10/0
1/64 *[1]	**9/0/0**	**0/0/9[#]**	**9/0/0**	**9/0/0**	**9/0/0**	**0/0/9[†]**	**9/0/0**	**9/0/0**	**9/0/0**	**9/0/0**	**8/2 (10/0)**
1/75 [3]	16/2/0	3/0/0	24/0/3	0/0/27	27/0/0	9/0/0	1/2/0	Pos[‡]	Pos[‡]	6/0/0	9/1
1/128 [1]	6/3/0	0/0/9	9/0/0	0/0/9	9/0/0	0/0/9	2/3/4	9/0/0	9/0/0	7/0/2	6/4
1/256 [1]	0/0/9	0/0/9	0/0/9	0/0/9	9/0/0	0/0/9	0/0/9	0/0/9	6/0/3	0/0/9	2/8
1/500 [2]	0/0/24	0/0/4	0/0/3	0/0/3	0/1/2	0/0/3	0/02	0/0/9	0/0/8	0/0/6	0/10
1/512 [1]	0/0/9	0/0/9	0/0/9	0/0/9	2/2/5	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	0/10
1/750 **[3]	**0/0/18**	**0/0/3**	**0/0/27**	**0/0/27**	**0/0/27**	**0/0/6**	**0/0/3**	**Neg[‡]**	**Neg[‡]**	**0/0/6**	**0/10**
1/1,024 [1]	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	0/10

1: Dilutions tested in first distribution round
2: Dilutions tested in second distribution round
3: Dilutions tested in third distribution round
#: Dilution negative in first round but in subsequent rounds 1/50 and 1/75 dilutions all classified as positive (3/0/0)
†: Dilution classified as negative in first round when bovine conjugate was used but 1/75 dilution classified as positive in third round when caprine conjugate was used
‡: Dilution not tested but previous data (round 1) indicate result

Table II
Competitive enzyme-linked immunosorbent assay results summary

Dilution	Test results									Overall
	A7	A8	B3	C9	E10	F8	G8	H7	I10	
Neat	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0
1/2	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0
1/4	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0
1/8 *	**9/0/0**	**3/0/6**	**9/0/0**	**9/0/0**	**9/0/0**	**9/0/0**	**9/0/0**	**9/0/0**	**9/0/0**	**8/0/1**
1/16	9/0/0	0/0/9	9/0/0	3/5/1	5/0/4	0/0/9	5/0/4	9/0/0	9/0/0	6/1/2
1/32	9/0/0	0/0/9	9/0/0	0/3/6	2/0/7	0/0/9	1/0/8	9/0/0	5/0/4	4/0/5
1/64	5/0/4	0/0/9	0/0/9	0/0/9	2/0/7	0/0/9	0/0/9	9/0/0	2/0/7	2/0/7
1/128	1/0/8	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	4/0/5	0/0/9	0/0/9
1/256	2/0/7	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9
1/300 **	NP	**Neg[‡]**	**Neg[‡]**	**Neg[‡]**	**Neg[‡]**	**Neg[‡]**	**Neg[‡]**	**Neg[‡]**	**Neg[‡]**	**0/0/9**
1/512	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9
1/1,024	1/0/8	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9	0/0/9

‡: Dilution not tested but previous data (round 1) indicate result
NP: test not performed

Table III
Fluorescent polarisation assay results summary

Dilution	Test results					Overall
	A1	B1	E1	H1	I1	
Neat	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	5/0/0
1/2	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	5/0/0
1/4	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	5/0/0
1/8	9/0/0	9/0/0	9/0/0	9/0/0	9/0/0	5/0/0
1/16*	**9/0/0**	**9/0/0**	**9/0/0**	**9/0/0**	**7/0/2**	**5/0/0**
1/32	8/0/1	9/0/0	5/4/0	8/1/0	0/0/9	4/0/1
1/64	0/5/4	0/6/3	0/2/7	4/2/3	0/0/9	0/2/3
1/128	0/0/9	0/2/7	0/0/9	2/1/6	0/0/9	0/0/5
1/200**	**Neg‡**	NP	**Neg‡**	NP	**Neg‡**	**0/0/5**
1/256	0/0/9	0/1/8	0/0/9	2/0/7	0/0/9	0/0/5
1/512	0/0/9	0/0/9	0/0/9	1/0/8	0/0/9	0/0/5
1/1,024	0/0/9	0/0/9	0/0/9	0/1/8	0/0/9	0/0/5

‡: Dilution not tested but previous data (round 1) indicate result
NP: test not performed

Table IV
Rose Bengal test results summary

Dilution	Test results								Overall
	A2	B3	D2	E5	E7	F6	G2	H7	
Neat	9/0	9/0	9/0	9/0	9/0	9/0	9/0	9/0	8/0
1/2	9/0	9/0	9/0	9/0	9/0	9/0	9/0	9/0	8/0
1/4	9/0	9/0	9/0	9/0	9/0	9/0	9/0	9/0	8/0
1/8	9/0	9/0	9/0	9/0	9/0	9/0	9/0	9/0	8/0
1/16*	**9/0**	**9/0**	**9/0**	**9/0**	**9/0**	**9/0**	**9/0**	**9/0**	**8/0**
1/32	9/0	9/0	9/0	6/3	9/0	9/0	0/9	9/0	7/1
1/64	4/5	0/9	9/0	0/9	0/9	9/0	0/9	9/0	3/5
1/128	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/8
1/200**	**Neg‡**	**Neg‡**	**Neg‡**	**Neg‡**	**Neg‡**	**Neg‡**	**Neg‡**	**Neg‡**	**0/8**
1/256	0/9	NP	NP	NP	NP	NP	NP	NP	0/1
1/512	0/9	NP	NP	NP	NP	NP	NP	NP	0/1
1/1,024	0/9	NP	NP	NP	NP	NP	NP	NP	0/1

‡: Dilution not tested but previous data (round 1) indicate result
NP: test not performed

The results for the modified RBT (mRBT) are shown in Table V. Only two laboratories performed this test, each with a different antigen. The results show that both data sets categorised the 1/32 and 1/64 pre-dilutions as positive on all occasions, and each categorised a 1/256 pre-dilution as negative on all occasions. Accordingly it was agreed that a 1/32 dilution should be the minimum analytical sensitivity threshold for the mRBT when testing the ISaBmS. To maintain a given safety margin it was agreed that the maximum analytical sensitivity threshold should not exceed a 1/400 dilution of the standard. No testing was performed at the 1/400 dilution but this value has been included in the table for indicative reasons, as described in the Discussion.

Table VI shows the dilutions of the ISaBmS that set the requirements for the minimum analytical sensitivity (min-ASn) and the lowest dilution that is less than the maximum analytical sensitivity (<max-ASn) for each of the tests, as democratically agreed by the authors of this paper.

Table V
Modified Rose Bengal test results summary

Dilution	Test results E5	Test results C11	Overall
Neat	3/0	9/0	2/0
1/2	3/0	9/0	2/0
1/4	3/0	9/0	2/0
1/8	3/0	9/0	2/0
1/16	3/0	9/0	2/0
1/32*	**3/0**	**9/0**	**2/0**
1/64	3/0	9/0	2/0
1/128	3/0	0/9	1/1
1/256	0/3	0/9	0/2
1/400**	**Neg‡**	**Neg‡**	NP
1/512	0/3	0/9	0/2
1/1024	NP	0/9	0/1

‡: Dilution not tested but previous data (round 1) indicate result
NP: test not performed

Table VI
Minimum requirements consensually agreed by the participating laboratories

Test	Minimum analytical sensitivity greater than or equal to this dilution	Maximum analytical sensitivity less than this dilution
iELISA	1/64	1/750
cELISA	1/8	1/300
FPA	1/16	1/200
RBT	1/16	1/200
mRBT	1/32	1/400

iELISA: indirect enzyme-linked immunosorbent assay
cELISA: competitive enzyme-linked immunosorbent assay
FPA: fluorescent polarisation assay
RBT: Rose Bengal test
mRBT: modified Rose Bengal test

Discussion

The availability of an international standard serum for standardising diagnostic tests is of importance in guaranteeing quality and providing confidence (19). Many diagnostic tests are highly effective when performed optimally, whereas suboptimal performance can lead to poor decision-making coupled with over-confidence. An international standard serum for use with serodiagnostic tests for bovine brucellosis has long been available (4), and this has helped to facilitate effective surveillance and transboundary trade. Although this serum has been, and is, used successfully to standardise tests that are not specific to an animal species (such as the RBT and CFT), tests that are specific for sheep and goats have not been standardised previously because of the lack of a specific international standard serum for small ruminants. The new ISaBmS solves this problem and it may also be used to standardise some tests that are not specific for a particular animal species when they are applied to sheep and goat samples.

This paper reports three important aspects. First, it provides provenance for the ISaBmS itself. Secondly, it shows the behaviour of the standard when tested with a range of commonly used brucellosis tests and test reagents. Finally, the minimum acceptable levels of standardisation to be met with the use of this serum have been agreed and defined by leading brucellosis diagnostic laboratories throughout the world.

The working criteria for the ISaBmS were defined in accordance with the principles already established for the OIEISS, whereby one standard is used to define positive and negative results (15), and for the OIEELISA$_{SP/WP/N}$SS as defined in Council Directive 64/432 (annex C) of the European Union (6), where dilutions of the standards are used to determine a limit to sensitivity.

The standardisation is achieved by defining minimum and maximum criteria for analytical sensitivity. In practical terms this is achieved by defining a dilution of the standard which, when tested by the particular diagnostic assay, results in a specified qualitative result. A minimum (min) standard for analytical sensitivity (ASn) is defined in this case by a specified pre-dilution (in negative serum) of the standard that must be categorised as positive (min-ASn). A maximum (max) standard for analytical sensitivity is defined by a specified pre-dilution (in negative serum) of the standard that must be categorised as negative (<max-ASn). Pre-dilutions within this range can be categorised as either positive or negative. Pre-dilutions outside this range should be categorised as negative if more dilute and positive if less dilute. The selection of the minimum and maximum sensitivity criteria was guided by a compromise between setting criteria that were demanding and informative and those that were regularly achievable by experienced laboratories worldwide using well-validated assays.

Whereas the requirement for a min-ASn value (dilution) is clear, the need for a <max-ASn value (dilution) is more debatable. In imperfect diagnostic assays increased sensitivity may result in decreased specificity. This is of particular relevance in the case of brucellosis where infection with Gram-negative bacteria possessing antigens of similar structure to *Brucella* can cause false-positive results in diagnostic tests (3). Therefore it has been considered reasonable to limit the analytical sensitivity of the different tests for brucellosis with the objective of minimising false-positive results. Analytical sensitivity is the ability of an assay to detect the presence of small quantities of analyte, and is distinct from diagnostic

sensitivity, which is the ability of the assay to identify samples from infected hosts correctly. This has been the main reason for establishing the maximum analytical sensitivity threshold. The drawback of this approach is that the use of such criteria may lead to future complications due to, for example, the introduction of new tests with greatly improved sensitivity and specificity. On balance the authors felt it was important to introduce criteria to assist in verifying specificity but with the reservation that this should not preclude the future introduction of new generation tests that do not meet these criteria but have extensive validation information to support their use, should such tests be developed.

It has been suggested previously that three standards should be used for iELISAs: a strong positive, a weak positive and a negative standard (20). There is certainly a strong case for the regular use of such quality control standards within each test. However, it is felt appropriate for the serodiagnosis of sheep and goat brucellosis by iELISA that a single standard can be used where specified pre-dilutions determine the minimum and maximum analytical sensitivity requirements. This is demonstrated in Figure 1, which shows representative ELISA results from three laboratories. Each has its own dose–response curve characteristic of that assay. However, each is able to differentiate between the 1/64 and 1/512 dilutions, enabling them to meet the criteria set for the iELISA.

This report establishes the criteria that must be met when using the ISaBmS to standardise the iELISA, cELISA and FPA. It also sets out supplementary criteria for the RBT that may be used in addition to the mandatory standardisation against the OIEISS (16). The dilutions that define these criteria have been established for each diagnostic test and are shown in Table VI. These values include some dilutions that were not actually included in the testing programme (i.e. the <max-ASn values for the cELISA, FPA, RBT and mRBT). These values were selected to strike a balance between two dilutions that were both tested but neither of which was felt to be exactly suitable, one being too dilute, the other too concentrated. The authors used their judgement, based on the evidence available from the existing results in addition to their considerable practical experience, to arrive at criteria that are demanding enough to establish a satisfactory minimum level of test performance yet deliverable on a regular basis in the laboratory. Where it was felt that additional evidence was required in order to make the correct choice additional dilutions were tested – as was the case for the iELISA.

The use of this standard will provide a very clear, transparent and measurable method of standardising tests. The use of sera calibrated to this standard could also assist in the quality control of reagent production and day-to-day test performance. All this is especially important for effective international trade testing and surveillance programmes. However, it is important to recognise that the defined criteria for the use of this standard represent only the minimum and maximum criteria for analytical sensitivity. Accordingly, their use in no way replaces or abrogates the requirement for all assays to be validated properly for diagnostic performance (i.e. with respect to diagnostic sensitivity and specificity), preferably in accordance with the OIE guidelines (14).

The test results obtained using different ampoules of the ISaBmS show that they were sufficiently uniform, because there were acceptably low levels of variation. The results from the nested ANOVA were particularly powerful because these were generated from data obtained from all the participating laboratories. The ANOVA result showed no evidence of any differences between the ampoules. The ANOVA also showed strong evidence of a difference in CFT results between days; however, the magnitude of this difference was inconsequentially small. The significant difference identified in the CFT results between participating laboratories shows that there are difficulties in the harmonisation of this technically demanding test. The data ranges were too large to be able to assign any criteria that were effective and could be universally agreed. Therefore, at least for the moment, the CFT will continue to be standardised by the OIEISS alone. This not only has implications regarding the use of the CFT for the international trade of animals but also raises questions about how a replacement standard for the OIEISS could be produced.

This standard and these criteria were recommended to the OIE Standards Commission for adoption and were accepted in September 2009. This standard should be used as a prototype for the production of national or secondary standards. It is hoped that its use will help to improve and regulate the quality of sheep and goat serodiagnosis and that this will help in turn to limit the spread of brucellosis.

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Premier sérum international de référence anti-*Brucella melitensis*

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Résumé

L'Organisation mondiale de la santé animale (OIE) a recommandé la mise au point d'un sérum international de référence anti-*Brucella melitensis* (ISaBmS) afin de standardiser les épreuves diagnostiques et les réactifs utilisés chez les ovins et caprins. Les critères sélectionnés ont été la dilution la plus élevée (d'un sérum négatif) avec laquelle on observe une réaction positive et la dilution la moins élevée (d'un sérum négatif) avec laquelle on observe simultanément une réaction négative. Les deux dilutions pour chaque épreuve ont été respectivement les suivantes : pour l'épreuve immuno-enzymatique indirecte (ELISAi), 1/64 et 1/750 ; pour l'ELISA de compétition (ELISAc), 1/8 et 1/300 ; pour l'épreuve de polarisation en fluorescence (EPF), 1/16 et 1/200 ; pour l'épreuve à l'antigène tamponné (EAT) ou rose Bengale, 1/16 et 1/200. Le sérum de référence international de l'OIE (OIEISS) doit continuer à être utilisé comme étalon primaire pour l'EAT, l'ISaBmS constituant un sérum de référence additionnel. Concernant l'épreuve de fixation du complément, il n'a pas été possible de définir des critères satisfaisants, de sorte que l'OIEISS reste l'étalon primaire. L'ISaBmS peut être utilisé pour standardiser les épreuves ELISAi, ELISAc et EAT pour le diagnostic de la brucellose ovine et caprine. Ce sérum de référence devrait faciliter l'harmonisation des épreuves utilisées pour la surveillance de la brucellose ou dans le cadre du commerce international de ces espèces.

Mots-clés

Brucella melitensis – Brucellose – Diagnostic – Sérologie – Standardisation.

Primer suero de referencia internacional anti-*Brucella melitensis*

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Resumen

La Organización Mundial de Sanidad Animal (OIE) pidió un suero de referencia internacional anti-*Brucella melitensis* (ISaBmS) con el fin de estandarizar los reactivos y las pruebas de diagnóstico para ovinos y caprinos. Los criterios acordados fueron: la dilución más alta (en suero negativo) del suero de referencia que debe dar resultado positivo y la dilución más baja (en suero negativo) que debe dar simultáneamente resultado negativo. En las distintas pruebas, esas diluciones fueron, respectivamente, las siguientes: ensayo inmunoenzimático indirecto (ELISAi), 1/64 y 1/750; ELISA de competición (ELISAc), 1/8 y 1/300; ensayo de fluorescencia polarizada (FP), 1/16 y 1/200; y prueba de rosa de Bengala (RB), 1/16 y 1/200. El suero de referencia

internacional de la OIE (OIEISS) seguirá siendo la referencia básica para la prueba RB, y el ISaBmS será en este caso una referencia complementaria. Resultó imposible definir criterios para la prueba de fijación del complemento, por lo que el OIEISS seguirá siendo el principal suero de referencia. Cabe utilizar el ISaBmS para estandarizar las pruebas ELISAi, ELISAc y FP de diagnóstico de la brucelosis en ovinos y caprinos. Este suero de referencia debería facilitar la armonización de las pruebas empleadas en la vigilancia de la brucelosis y en el comercio internacional de dichas especies.

Palabras clave
Brucella melitensis – Brucelosis – Diagnóstico – Estandarización – Serología.

References

1. Benkirane A. (2006). – Ovine and caprine brucellosis: world distribution and control/eradication strategies in West Asia/North Africa region. *Small Rum. Res.*, **62**, 19–25.

2. Blasco J.M. (2010). – A critical step for decision makers: control or eradication of *Brucella melitensis*? In *Brucella melitensis* in Eurasia and the Middle East. FAO technical meeting in collaboration with WHO and the OIE, May 2009, Rome. Animal Production and Health Division, Food and Agriculture Organization of the United Nations, Rome.

3. Corbel M.J. (1985). – Recent advances in the study of *Brucella* antigens and their serological cross-reactions. *Vet. Bull.*, **55**, 927–942.

4. Davidson I., Herbert C.N. & Brinley Morgan W.J.N. (1969). – The Second International Standard for anti-*Brucella abortus* Serum. *Bull. WHO*, **40**, 129–140.

5. European Food Safety Authority/European Centre for Disease Prevention and Control (2009). – The Community summary report on trends and sources of zoonoses and zoonotic agents in the European Union in 2007. *EFSA J.*, **223**, 192.

6. European Union (2008). – Commission Decision 2008/984/EC of 10 December 2008 amending Annex C to Council Directive 64/432/EEC and Decision 2004/226/EC as regards diagnostic tests for bovine brucellosis. *Off. J. Eur. Union*, 31/12/2008, **L 352**, 38–45.

7. Miller G.E. (1991). – Asymptotic test statistics for coefficients of variation. *Communic. Statist. Theor. Meth.*, **20**, 2251–2262.

8. Murray P.R. & Corbel M. (2007). – *Brucella*. *In* Topley and Wilson's microbiology and microbial infections: bacteriology (S.P. Boriello, P.R. Murray & G. Funke, eds), 10th Ed. Edward Arnold, London, 1719–1751.

9. Palmer D.A. & Douglas J.T. (1989). – Analysis of *Brucella* lipopolysaccharide with specific and cross-reacting monoclonal antibodies. *J. clin. Microbiol.*, **27** (10), 2331–2337.

10. Pappas G., Papadimitriou P., Akritidis N., Christou L. & Tsianos E.V. (2006). – The new global map of human brucellosis. *Lancet infect. Dis.*, **6**, 91–99.

11. Refai M. (2002). – Incidence and control of brucellosis in the Near East region. *Vet. Microbiol.*, **90**, 81–110.

12. Robinson A. (2010). – *Brucella melitensis* infections: food-borne versus animal contact. *In Brucella melitensis* in Eurasia and the Middle East. FAO technical meeting in collaboration with WHO and the OIE, May 2009, Rome. Animal Production and Health Division, Food and Agriculture Organization of the United Nations, Rome.

13. World Health Organization (WHO) (1953). – WHO Technical Report Series No. 68. Sixth report of the WHO Expert Committee on Biological Standardization. WHO, Geneva. Available at: http://whqlibdoc.who.int/trs/WHO_TRS_68. pdf.

14. World Organisation for Animal Health (OIE) (2008). – Chapter 1.1.4.: Principles of validation of diagnostic assays for infectious diseases. *In* Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 6th Ed. OIE, Paris, 34–45.

15. World Organisation for Animal Health (OIE) (2008). – Chapter 2.4.3.: Bovine brucellosis. *In* Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 6th Ed. OIE, Paris, 624–659.

16. World Organisation for Animal Health (OIE) (2008). – Chapter 2.7.2.: Caprine and ovine brucellosis (excluding *Brucella ovis* infection). *In* Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 6th Ed. OIE, Paris, 974–982.

17. World Organisation for Animal Health (OIE) (2008). – OIE Guide 3: International reference antibody standards for antibody assays. *In* OIE quality standard and guidelines for veterinary laboratories: infectious diseases, 2nd Ed. OIE, Paris, 45–52.

18. World Organisation for Animal Health (OIE) (2009). – Chapter 14.1.: Caprine and ovine brucellosis (excluding *Brucella ovis*). *In* OIE Terrestrial Animal Health Code, 18th Ed. OIE, Paris, 679–684.

19. Wright P.F. (1998). – International standards for test methods and reference sera for diagnostic tests for antibody detection. *In* Veterinary laboratories for infectious diseases (J.E. Pearson, ed.). *Rev. sci. tech. Off. int. Epiz.*, **17** (2), 527–533.

20. Wright P.F., Tounkara K., Lelenta M. & Jeggo M.H. (1997). – International reference standards: antibody standards for the indirect enzyme-linked immunosorbent assay. *Rev. sci. tech. Off. int. Epiz.*, **16** (3), 824–832.