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Capripox test validation

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Abstract

Capripoxviruses cause sheeppox, goatpox, and lumpy skin disease of cattle, which are the most serious poxvirus diseases of production animals. There are currently no validated, high-throughput antibody detection tools available for undertaking post-outbreak surveillance and re-establishing freedom from disease, which is the primary factor that would mitigate the economic impact to livestock industries in the event of an outbreak. This project builds directly on a previous MDC-funded project, which led to the development and preliminary validation of an antibody detection ELISA for capripoxviruses. To determine the reproducibility of this assay, an inter-laboratory evaluation of the ELISA was undertaken. Useable data were received from five out of eight participating laboratories (in Australia, North America, Europe and Africa), all of which correctly classified each test sample as positive or negative. Based on the analysis of paired samples in each serum panel, acceptable reproducibility and repeatability were demonstrated. Subsequent analysis identified the ELISA blocking buffer as a likely contributor to the variation observed in the results. This buffer will need to be replaced prior to test implementation internationally, and this would be best undertaken by conversion of the ELISA into kit form in collaboration with a commercial partner.

Executive summary

Sheeppox, goatpox, and lumpy skin disease are the most serious poxvirus diseases of production animals. Although the risk of these diseases entering Australia is considered low, the potential economic impact of an incursion would be considerable, most likely second only to that anticipated following an introduction of foot-and-mouth disease. A capripoxvirus disease incursion would potentially cause considerable economic losses due to disruption of trade in livestock and livestock products, as well as costs associated with disease control and eradication. Significantly, there are currently no validated, high-throughput serodiagnostic tools available for undertaking post-outbreak surveillance and re-establishing freedom from disease, which is the primary factor that would mitigate the economic impact to the livestock industries in the event of an outbreak. Importantly the current "gold standard" serum neutralisation test has not been standardised anywhere in the world, is not suitable for high-throughput antibody detection, and cannot be implemented at the Australian Animal Health Laboratory (AAHL) as it requires the use of live virus, which is not available in Australia (AAHL is not permitted to hold or import live virus).

This project builds directly on a previous MLA Donor Company funded project (P.PSH.0623), the key outcome of which was the development and preliminary validation of a high-throughput serodiagnostic test (ELISA) for capripoxviruses. With the potential global application of high throughput tests for these diseases, OIE principles, methods and guidelines for test development and validation were followed. This newly developed ELISA, which is based on a purified viral recombinant protein, designated Cp 25, offers the prospect of a reliable and standardised high-throughput antibody detection capability, without the requirement for infectious reagents, to support post-outbreak surveillance and proof of freedom testing should a capripoxvirus outbreak occur in the future. To determine the reproducibility of the assay, which is the ability of the ELISA to provide consistent results when applied to aliquots of the same samples tested in different laboratories, by different operators, using the identical protocol, reagents and controls, an inter-laboratory evaluation of the ELISA was conducted. Determination of reproducibility is a key condition in the final diagnostic test validation pathway, and is required to obtain international acceptance and recognition of the test according to OIE guidelines. With assistance from collaborators in Canada, well-characterised panels of sheep, goat and cattle test and control sera were prepared for this purpose, and their reactivity and homogeneity confirmed prior to distribution to the overseas collaborating laboratories. Of eight laboratories that participated in the evaluation of the ELISA, five returned data that were acceptable for comparative analysis.

Each laboratory correctly classified all 28 blind coded cattle, sheep and goat sera as positive or negative, representing 100% agreement between the participating laboratories based on qualitative interpretation of the ELISA data. However, considerable variation was observed between the laboratories in the values obtained for the high and low positive control sera. Despite the suboptimal performance of the ELISA in several laboratories, this did not result in misclassification of any of the test samples. Furthermore, based on statistical analysis of the data derived from the blind coded paired sera that were included in each serum panel, acceptable reproducibility and repeatability of the assay were demonstrated. Outlying results (where there was significantly greater variation in the paired data for specific laboratories compared to the median of all participating laboratories) were

obtained for only three samples and, in each of these instances, by only one of the five participating laboratories.

Subsequent review of the reagents and buffers that are utilised in the ELISA protocol identified the commercial casein-based blocking buffer as a likely contributor to the between-laboratory variation observed in the data. This particular buffer will need to be replaced prior to implementation of the assay internationally and, based on the results shown in this report, this would be best undertaken by conversion of the ELISA into kit form in collaboration with a commercial partner.

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1 Background

Capripoxviruses cause sheeppox, goatpox and lumpy skin disease of cattle, which are the most serious poxvirus diseases of production animals. Although the risk of these diseases entering Australia is considered low (Australia has never had an outbreak), the potential economic impact of an incursion would be considerable; for example an outbreak of sheeppox would likely be second to that anticipated following an introduction of foot-and-mouth disease (Garner and Lack 1995). Contributing factors would include the resultant disruption to trade in livestock and livestock products (meat, leather and wool), the impact of the diseases on animal health and welfare, and costs associated with disease control and eradication. Significantly, however, there are currently no validated, high-throughput serodiagnostic tools available for undertaking post-outbreak surveillance and re-establishing freedom from disease, which is the primary factor that would mitigate the economic impact to the livestock industries in the event of an outbreak.

The Australian Animal Health Laboratory (AAHL) recently led a major international collaborative project (2004 - 2007, with funding from CSIRO and AWI), with the Canadian National Centre for Foreign Animal Disease (NCFAD), to enhance the diagnostic capability for these diseases. As a result of this work new international collaborations were established to work on these long neglected viruses, and AAHL now has in place a set of updated tests and reagents that has significantly enhanced its emergency response capability for the primary diagnosis of these diseases (Babiuk *et al.* 2007; Babiuk *et al.* 2008; Bowden *et al.* 2008; Babiuk *et al.* 2009a; Embury-Hyatt *et al.* 2012; Stubbs *et al.* 2012). These assays and methods include electron microscopy, histology, immunohistochemistry and quantitative real-time PCR, as well as the capability to isolate capripoxviruses in cell culture if required to do so in future disease outbreak investigations.

In contrast, the serum neutralisation test, considered the gold standard for antibody detection, has not been standardised anywhere in the world, is not suitable for high-throughput testing, and cannot be implemented at AAHL since it requires live virus, and importation and holding live virus is prohibited in Australia.

To address the deficit in AAHL's serodiagnostic capability for capripoxviruses, development and preliminary evaluation of an ELISA based on recombinant antigens derived from sheeppox virus was undertaken during the AWI-funded project. Although this assay initially appeared suited to detecting antibodies in infected sheep, goats and cattle (Bowden *et al.* 2009), additional validation (partially supported by MLA), using cattle sera from outbreaks of lumpy skin disease in Africa, subsequently demonstrated that it does not provide the desired diagnostic sensitivity and specificity, particularly for cattle. An indirect ELISA, which uses inactivated, sucrose gradient-purified sheeppox virus as coating antigen, was also developed (Babiuk *et al.* 2009b). However, although suited to screening sera from sheep, goats and cattle, the viral antigen is difficult and expensive to produce in large quantities. Furthermore, its production requires access to live virus, which is not available in Australia.

To identify alternative candidate antigens for improved test development, CSIRO subsequently funded a collaboration with colleagues at the University of California, Irvine (UC Irvine), who had pioneered a high-throughput cloning and protein microarray chip fabrication pipeline that is ideally suited to profiling immunoreactivity on a large scale (Davies *et al.* 2005). Fabrication and screening

of chips comprising all of the proteins (~450) encoded by sheeppox, goatpox and lumpy skin disease viruses shed new insights into the host immune response following exposure, and clearly demonstrated the utility of this technology for identification of immunodominant antigens from complex microorganisms in an efficient and straightforward manner. A subset of antigens that appeared to be broadly reactive in sheep, goats and cattle were identified, and these were preferred potential candidates for development of an improved antibody detection ELISA to enhance Australia's disease preparedness, and support control and eradication programs.

Subsequently, with funding from CSIRO and the MLA Donor Company (Project No. P.PSH.0623), multiple antibody detection ELISA tests, based on these recently discovered immunodominant viral antigens, were developed and evaluated using sera from experimentally or naturally infected sheep, goats and cattle. The three most promising assays were chosen for more extensive evaluation at AAHL (Australia), NCFAD (Canada) and the Capripoxvirus Reference Laboratory at The Pirbright Institute (UK). Although all three exhibited differential reactivity to sheep, goat and cattle sera, one antigen consistently demonstrated broader and stronger reactivity, identifying it as the lead candidate for ongoing ELISA validation activities. This ELISA performed favourably when sera from sheep, goats or cattle infected naturally or experimentally with virulent capripoxvirus isolates were tested, with preliminary estimates of diagnostic sensitivity and specificity ranging between 98 and 100%, but it was unable to detect antibodies reliably in vaccinated sheep, goats or cattle. Nevertheless, this appears to be a deficiency encountered with all currently available assays for capripoxvirus serodiagnosis including the serum neutralisation test.

This project builds directly on the previous MDC-funded project (P.PSH.0623), the main outcome of which was the development and preliminary validation of an ELISA for the diseases caused by capripoxviruses (sheeppox, goatpox and lumpy skin disease). The purpose of the current project was to determine the reproducibility of the assay, which is the ability of the ELISA to provide consistent results when applied to aliquots of the same samples tested in different laboratories, by different operators, using the identical protocol, reagents and controls. Determination of assay reproducibility is a critical component of the OIE assay validation pathway that needs to be undertaken for the test to be considered as "validated for the original intended purpose".

2 Project objectives

The project objective is to determine the reproducibility of the ELISA in at least three different laboratories, located in separate countries, by testing the same three panels of blind coded sera (one each of sheep, goat and cattle origin). Determination of assay reproducibility fulfils a key condition in the diagnostic test validation pathway and is required to subsequently obtain international acceptance and recognition of the test according to OIE guidelines, which would be vital for re-establishing disease free status and expediting the resumption of trade in livestock and livestock products should the ELISA be used in the event of an outbreak in Australia.

Within 6 months

- Confirm involvement of participating overseas laboratories (from Europe, North America or Africa) under appropriate agreements.
- Prepare and confirm reactivity of well-characterised control sheep, goat and cattle sera for distribution to overseas collaborating laboratories (in conjunction with the Capripoxvirus Reference Laboratory at The Pirbright Institute (UK) and the National Centre for Foreign Animal Disease (Canada)).
- Prepare and confirm reactivity of well-characterised test sheep, goat and cattle sera for distribution to overseas collaborating laboratories (in conjunction with the Capripoxvirus Reference Laboratory at The Pirbright Institute (UK) and the National Centre for Foreign Animal Disease (Canada)). Sera from sheep, goats or cattle would be included in each evaluation panel and would be blind coded prior to distribution.

Within 12 months

- Distribute the control sera, evaluation panels, and all required ELISA materials and reagents to participating laboratories (in conjunction with the Capripoxvirus Reference Laboratory at The Pirbright Institute (UK)).
- Coordinate the inter-laboratory evaluation of the ELISA using the sheep, goat and cattle serum panels.
- Collate and analyse data, and determine assay reproducibility.

3 Methodology

3.1 Participating laboratories

In addition to the Diagnostic Emergency Response Laboratory (DERL), located at CSIRO AAHL in Geelong, seven overseas laboratories, located in North America, Europe and Africa, were invited to participate in the inter-laboratory evaluation of the Capripox Cp 25 ELISA. All are national / government laboratories with responsibilities that include diagnostic testing for, and scientific research on, sheeppox, goatpox and lumpy skin disease viruses. The details of the participating laboratories are as follows:

<u>Australia</u>

1. CSIRO Australian Animal Health Laboratory (AAHL) Geelong, Australia.

<u>North America</u>

1. National Centre for Foreign Animal Disease (NCFAD), Winnipeg, Canada.

Europe

1. OIE Reference Laboratory for sheeppox, goatpox and lumpy skin disease, The Pirbright Institute, UK.
2. Veterinary and Agrochemical Research Centre (CODA-CERVA), Brussels, Belgium.
3. Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Montpellier, France.
4. International Atomic Energy Agency (IAEA) Laboratories Seibersdorf, Seibersdorf, Austria.

Africa

1. OIE Reference Laboratory for lumpy skin disease, Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI), Onderstepoort, South Africa.
2. National Animal Health and Diagnostic Investigation Centre (NAHDIC), Sebeta, Ethiopia.

3.2 Preparation of serum panels

Selection of the control and test cattle, sheep and goat sera encompassing the serum evaluation panels (including strong, medium and weak positive, as well as negative, sera) was based on data generated during prior testing conducted under the previous
MDC-funded Capripox ELISA Project (P.PSH.0623). Since larger sample volumes were held within the serum collection at NCFAD, Canada, sera from NCFAD rather than from The Pirbright Institute (UK) were utilised for preparation of the serum panels. This work was undertaken at NCFAD between 27 February and 21 March 2016, following transfer of Cp 25 antigen to the Canadian laboratory. All test and control sera were evaluated using the Cp 25 ELISA and their expected reactivity confirmed.

Due to the number of participating overseas laboratories, and to simplify logistics, the final packing, testing and distribution of the serum panels, which had to be shipped on dry ice, were coordinated and managed directly from AAHL. All sera were therefore gamma irradiated at 5 MRad on entry to Australia, as per the import permit conditions. Gamma irradiation of the samples allowed a greater number of participants to be included in the inter-laboratory evaluation of the assay, as not all were able to receive potentially infectious samples. All test samples were blind coded prior to distribution.

3.3 Homogeneity testing

Homogeneity testing was conducted following recommended guidelines (Thompson *et al.* 2006). Replicate 100 µl aliquots of the control and test sera were made in 1.5 ml O-ring capped tubes (Sartstedt) and stored at -20°C. Ten replicates of each control and test sample were selected at random using a random number generator in Microsoft Excel. The samples were thawed at room temperature, vortexed briefly and clarified by centrifugation at 13,000 rpm for 1 minute in a benchtop microcentrifuge. For each control and test serum two test portions from all ten randomly selected tubes were subsequently tested on the same ELISA plate, at a dilution of 1:100, in duplicate, using the Cp 25 ELISA protocol (summarised in Appendix 1). For each serum sample, the coefficient of variation was determined by dividing the between-samples (between-replicates) standard

deviation by the mean of all replicates tested. Samples were considered to be of sufficient homogeneity if the coefficient of variation was less than 15%.

3.4 Storage and shipment of samples

The required temperature for long term storage of the Cp 25 ELISA antigen is -80°C, while the replicate aliquots of test and control sera were stored at -20°C following preparation. Due to the requirement for the Cp 25 ELISA antigen to remain frozen prior to use, the antigen and sera were shipped to the participating laboratories on dry ice (under appropriate country-specific import permits). The participants were asked to confirm receipt of the samples and their condition when received. The participating laboratories were also instructed to ensure that the samples remained frozen on arrival (-80°C for the antigen and
-20°C for the sera), until thawed for testing.

3.5 Supply of reagents

Where possible, all participating laboratories were requested to source reagents locally, as the intention was to evaluate the assay under the conditions that it would be used if and when implemented for ongoing use. In the absence of a commercialisation contract it was envisioned that the ELISA would be implemented by distribution of the Cp 25 protein from an AAHL stock, with all other reagents supplied by the end user. All relevant catalogue numbers, and comprehensive instructions for reagent and buffer preparation, were provided as part of the inter-laboratory evaluation test protocol.

The only exception was NAHDIC (Ethiopia), for whom sourcing the required reagents was problematic. All assay reagents, buffers and consumables were therefore shipped to NAHDIC, either at room temperature or on frozen gel packs, along with the antigen and sera on dry ice.

3.6 Test procedure

All participating laboratories were sent comprehensive instructions on how to implement the ELISA and test the cattle, sheep and goat serum panels (refer to Appendix 1 for details).

3.7 Data collection

For each species (bovine, ovine and caprine), mean OD values were calculated for the control and test (blind coded) sample wells. These values were corrected by subtracting the mean OD value of the negative control reference serum of each species (BC-, OC- or CC-) from the mean OD value of the respective control and test samples. To enable comparison of data between ELISA plates, within and between laboratories, the results were normalised against the low positive control serum and expressed as an S/P ratio (the ratio of the corrected mean OD value of the test sample (S) to the corrected mean OD value of the low positive control (P) reference serum (BC+, OC+ or CC+, respectively)), as described in Appendix 1. These calculations, and those pertaining to the test controls, were performed automatically when the raw OD values were entered into an Excel data template (protected to prevent inadvertent deletion of embedded formulae) that was provided to all participants. The completed Excel worksheets were returned to AAHL for further analysis.

Participants were instructed to provide lot and batch numbers of all reagents and buffers used to assist with troubleshooting should there be difficulties encountered in implementation of the assay, or should data from particular laboratories differ significantly from the others. Data from laboratories that did not conduct testing as instructed were excluded from the comparative analyses.

3.8 Data analysis, summary statistics and Z-scores

After reviewing raw and transformed data submitted by the participating laboratories to check for potential errors, robust summary statistics were used for analysis of the results, since they are not influenced by the presence of outliers in the data (Proficiency Testing Australia 2016). These included the median, normalised interquartile range (IQR_N), robust coefficient of variation (CV), minimum, maximum and range, and are defined below (Proficiency Testing Australia 2016).

The median is the middle value of the data set for each sample tested.

The normalised IQR is a measure of the variability of the results, and is equal to the interquartile range (IQR) multiplied by a correction factor (0.7413), which makes it comparable to a standard deviation (where the interquartile range is the difference between the lower and upper quartiles).

$$\mathrm{IQR_N} = \mathrm{IQR} \times 0.7413$$

The robust CV is a coefficient of variation that is equal to the normalised IQR divided by the median, expressed as a percentage, and it allows for the variability in the different samples to be compared.

$$\mathrm{CV} = 100 \times \left(\frac{\mathrm{IQR_N}}{\mathrm{Median}}\right)$$

The minimum is the lowest value in the data set for each sample, the maximum is the highest value and the range is the difference between them.

Although useable data for comparison was only obtained from five of the participating laboratories, Z-scores, based on robust summary statistics, were calculated for the related sample pairs that were present in each species-specific panel to facilitate the evaluation of both the between-laboratory and within-laboratory variation in the data generated using the ELISA. These included not only the identical pairs, for which the results would be expected to match, but also the split pairs, for which the results should only be slightly, but uniformly, different (Proficiency Testing Australia 2016).

The between-laboratories Z-score and the within-laboratory Z-score are usually based on the sum and difference of the pair of results, respectively, and are defined as follows (Proficiency Testing Australia 2016).

The standardised sum (S) and standardised difference (D) for a pair of results, say from two samples labelled A and B, are:

$$S = \frac{(A + B)}{\sqrt{2}}$$

$$D = \frac{(A - B)}{\sqrt{2}} \qquad \text{or, if } A < B \qquad D = \frac{(B - A)}{\sqrt{2}}$$

Each laboratory's standardised sum and difference are calculated, followed by the median and normalised IQR of all of the S and D calculations (median(S), $IQR_N(S)$, median(D) and $IQR_N(D)$).

The between-laboratory Z-score (ZB) is then calculated as the robust Z-score for S

$$ZB = \frac{S - median(S)}{IQR_N(S)}$$

while the within-laboratory Z-score (ZW) is the robust Z-score for D

$$ZW = \frac{D - median(D)}{IQR_N(D)}$$

However, for the identical sample pairs the assumption was that the results obtained by the participating laboratories, for each sample pair, should be the same. The data for these samples were therefore assessed against a standardised difference of zero when determining the within-laboratory variation.

The interpretation of the Z-scores is as follows: $|Z| \leq 2.0$ is considered acceptable; $2.0 < |Z| < 3.0$ indicates "questionable" performance; and $|Z| \geq 3.0$ indicates an "unsatisfactory" performance (the result is an outlier) (Thompson *et al.* 2006; Proficiency Testing Australia 2016).

Youden diagrams were also generated for each related sample pair. Each laboratory's pair of results was plotted, together with an approximate 95% confidence ellipse for the bivariate analysis of the results (Proficiency Testing Australia 2016), and with the median value of each sample being illustrated by dashed lines perpendicular to the x- and y-axes. The diagrams were used to assess for the presence of significant systematic error (between-laboratories variation), where data points would be located outside the ellipse in either the upper right hand quadrant or the lower left hand quadrant (unexpectedly high or low results for both samples), as well as significant random error (within-laboratory variation), where data points would be located outside the ellipse in either the upper left or lower right hand quadrant (unexpectedly high result for one sample and low for the other) (Proficiency Testing Australia 2016).

4 Results

4.1 Panel composition

Three separate panels of sera, comprising either bovine, ovine or caprine samples, were constructed using samples that had been previously characterised at NCFAD, Canada, during the prior development and evaluation of the Cp 25 ELISA. The control sera comprised high and low positive, as well as negative, sera (C++, C+ and C-, respectively), while the test sera included representative high, medium and low positive, as well as negative, sera. Amongst the negative sheep and goat test sera were included orf virus positive, but capripoxvirus negative, samples to allow evaluation of assay specificity. In addition, paired sera were included in each panel to facilitate assessment of both within and between laboratory variation of the assay. These sera constituted either identical pairs, where the sera were replicate aliquots of the same sample, or split pairs, where one serum sample was generated by dilution of the other (by a factor of between 1.2 and 1.5) in normal cattle, sheep or goat serum. None of the relationships between specific sera were disclosed to the participants, so that each sample would be treated independently of the other.

For cattle the test panel comprised two negative sera and six positive sera, including one identical pair and two split pairs (Table 1). Although not distributed as a control sample tube, no serum (diluent only) wells were included in the bovine panel (see Fig. 2 in Appendix 1) to enable background reactivity of the assay to be determined.

Table 1. Composition of bovine serum panel.

Bovine sample	Expected Result	Relationship	Purpose
C++	High positive		Positive control
C+	Low positive		Positive control (data normalisation)
C-	Negative		Negative control
No serum	Negative		Assess background
1	Low positive	Identical to 8	Assess repeatability & reproducibility
2	Negative	Single sample	Assess contamination
3	Low positive	Dilution of 1	Assess repeatability & reproducibility
4	High positive	Related to 6	Assess repeatability & reproducibility
5	High positive	Single sample	Representative high positive
6	Medium positive	Dilution of 4	Assess repeatability & reproducibility
7	Negative	Single sample	Assess contamination
8	Low positive	Identical to 1	Assess repeatability & reproducibility

For sheep the test panel comprised two negative sera and eight positive sera, including two identical pairs and two split pairs (Table 2).

Table 2. Composition of ovine serum panel.

Ovine sample	Expected result	Relationship	Purpose
C++	High positive		Positive control
C+	Low positive		Positive control (data normalisation)
C-	Negative		Negative control
1	Medium positive	Identical to 5	Assess repeatability & reproducibility
2	Low positive	Related to 4	Assess repeatability & reproducibility
3	Negative	Single sample	Assess contamination
4	Low positive	Dilution of 2	Assess repeatability & reproducibility
5	Medium positive	Identical to 1	Assess repeatability & reproducibility
6	High positive	Related to 8	Assess repeatability & reproducibility
7	High positive	Identical to 9	Assess repeatability & reproducibility
8	Medium positive	Dilution of 6	Assess repeatability & reproducibility
9	High positive	Identical to 7	Assess repeatability & reproducibility
10	Negative	Orf virus positive	Assess specificity & contamination

For goats the test panel comprised two negative sera and eight positive sera, including two identical pairs and one split pair (Table 3).

Table 3. Composition of caprine serum panel

Caprine sample	Expected result	Relationship	Purpose
C++	High positive		Positive control
C+	Low positive		Positive control (data normalisation)
C-	Negative		Negative control
1	Low positive	Identical to 8	Assess repeatability & reproducibility
2	Medium positive	Related to 7	Assess repeatability & reproducibility
3	Negative	Orf virus positive	Assess specificity & contamination
4	High positive	Single sample	Representative high positive
5	Negative	Single sample	Assess contamination
6	High positive	Single sample	Representative high positive
7	Low positive	Dilution of 2	Assess repeatability & reproducibility
8	Low positive	Identical to 1	Assess repeatability & reproducibility
9	Medium positive	Identical to 10	Assess repeatability & reproducibility
10	Medium positive	Identical to 9	Assess repeatability & reproducibility

Although the cattle, sheep and goat control and test sera comprised independent panels for evaluation of the ELISA as three separate assays, the number and composition of samples were chosen to fill one complete 96-well ELISA plate such that all control sera were tested in quadruplicate, and all blind coded (unknown) test sera in duplicate (Fig. 2, Appendix 1).

4.2 Homogeneity testing

Following confirmation of the expected reactivity of all control and test sera, replicate 100 µl aliquots of each sample were made in 1.5 ml O-ring capped tubes and stored at -20°C. The control sera were clearly labelled, while the replicate test samples were blind coded (Bovine sample 1 to 8, Ovine sample 1 to 10 and Caprine sample 1 to 10), so that the relationships between them could not be determined by the participants.

Ten randomly selected replicate aliquots of each control and test serum were tested, in duplicate, and on the same ELISA plate, using the Cp 25 assay to confirm that they were sufficiently homogeneous. For all sera, the coefficient of variation between replicate samples was less than 5%, except for Caprine sample 3, which had a coefficient of variation of 6.39% (Table 4). All control and

test sera were therefore confirmed to be homogeneous (below an upper threshold of 15%) prior to shipping.

Table 4. Homogeneity testing of bovine, ovine and caprine control and test sera.
Abbreviations: OD (optical density); SD (standard deviation); CV (coefficient of variation).

Bovine	Mean OD	SD	CV (%)
C++	1.405	0.000	0.00
C+	0.520	0.015	2.88
C-	0.064	0.001	1.18
1	0.589	0.011	1.83
2	0.057	0.001	0.97
3	0.378	0.000	0.00
4	1.513	0.016	1.04
5	2.238	0.070	3.12
6	1.203	0.048	3.97
7	0.054	0.002	4.54
8	0.609	0.021	3.46

Ovine	Mean OD	SD	CV (%)
C++	1.310	0.036	2.78
C+	0.459	0.000	0.00
C-	0.057	0.001	0.99
1	1.142	0.025	2.19
2	0.530	0.000	0.00
3	0.052	0.0004	0.80
4	0.355	0.007	1.92
5	1.119	0.023	2.06
6	1.783	0.059	3.33
7	2.135	0.047	2.19
8	0.922	0.000	0.00
9	2.145	0.054	2.51
10	0.059	0.002	2.67

Caprine	Mean OD	SD	CV (%)
C++	1.441	0.000	0.00
C+	0.534	0.008	1.59
C-	0.068	0.001	1.60
1	0.416	0.007	1.61
2	0.985	0.017	1.68
3	0.083	0.005	6.39
4	2.189	0.000	0.00
5	0.067	0.001	2.13
6	1.648	0.000	0.00
7	0.791	0.024	2.99
8	0.420	0.000	0.00
9	1.469	0.000	0.00
10	1.463	0.000	0.00

4.3 Sample transport

All consignments were shipped using the same international air courier. All samples were confirmed by the participating laboratories to have arrived in good order, with samples arriving frozen on dry ice. The one exception was NAHDIC in Ethiopia. Despite spending several months obtaining the required paperwork and permits (coordinated by the international courier), the consignment subsequently took 3 months to clear customs following its arrival in Addis Ababa. There was no opportunity for the antigen or sera to be stored frozen (at -80°C or -20°C) during this time. However, the box containing antigen and sera (on dry ice) was reportedly stored refrigerated (4°C), along with the box of reagents and buffers that had been shipped on frozen gel packs (for storage on arrival at 4°C). Due to project time limitations and uncertainty that customs clearance would proceed more rapidly for a future shipment, it was agreed that the testing at NAHDIC would proceed rather than resend additional aliquots of antigen and sera.

4.4 Test implementation

Of the eight laboratories that participated in the evaluation of the ELISA, three were excluded from the subsequent analyses. The first, although it correctly classified all test cattle, sheep and goat sera as positive or negative, did not conduct the test as instructed and therefore direct comparisons with data generated by the other laboratories could not be undertaken. Neither of the other two laboratories was able to obtain clear differentiation between the C++, C+ or C- sera, making classification of the test sera impossible. One of these two labs was NAHDIC in Ethiopia, for which the antigen and sera had not been stored appropriately during the 3 months that it took for the consignment to clear customs. The other laboratory was only able to test a single plate, and the reason for the assay failure in this laboratory was not readily apparent.

Data were received back from the remaining 5 laboratories (designated A - E and located in Australia, North America, Europe and Africa), all of which correctly classified each test sample as positive or negative. However, as illustrated in Table 5, considerable variation was noted, between the laboratories, in the values obtained for the high and low positive control sera. For the cattle control sera three laboratories (B, C & D) were within the expected range for the low positive sample, while lab A was just above, and lab E just below, the expected range. Only two labs (B and E) had the S/P ratio of the high positive control serum within the expected range, while the remaining four were just below the lower limit.

The pattern was very similar for the sheep controls with three laboratories (B, C & E) falling within the expected range for the low positive sample, while two labs (A and D) were just outside the upper limit of the expected range. Two labs (B and E) had the S/P ratio of the high positive control serum within the expected range, while the remaining three were just under the lower limit (Table 5).

For the goat control sera three laboratories (B, C & E) fell within the expected range for the low positive sample, while the other two labs were well above the upper limit of the expected range. Laboratories B, C and E had the S/P ratio of the high positive control serum fall within the expected range, while the other two were well below the lower limit.

Table 5. Comparison of OD values and S/P ratios obtained for control sera when tested in the Cp 25 ELISA by the participating laboratories. Values falling within the expected range are indicated in bold.

Sample	Expected range	Actual result by laboratory				
		A	B	C	D	E
BC+	0.30 to 0.90	0.95	**0.50**	**0.77**	**0.85**	0.26
BC-	<0.20	**0.15**	**0.07**	**0.16**	**0.08**	**0.11**
BC++	2.6 to 3.4	2.3	**2.8**	2.4	2.4	**2.8**
OC+	0.30 to 0.90	1.04	**0.42**	**0.75**	1.03	**0.31**
OC-	<0.20	**0.12**	**0.05**	**0.10**	**0.07**	**0.09**
OC++	2.7 to 3.7	2.3	**3.0**	2.6	2.4	**3.0**
CC+	0.30 to 0.90	1.51	**0.50**	**0.45**	1.98	**0.36**
CC-	<0.20	**0.17**	**0.07**	**0.17**	**0.08**	**0.12**
CC++	2.5 to 3.4	1.8	**2.7**	**2.5**	1.7	**2.8**

Nevertheless, as depicted in Fig. 1, all five laboratories correctly classified all 28 test sera as either positive or negative based on the calculated S/P ratios.

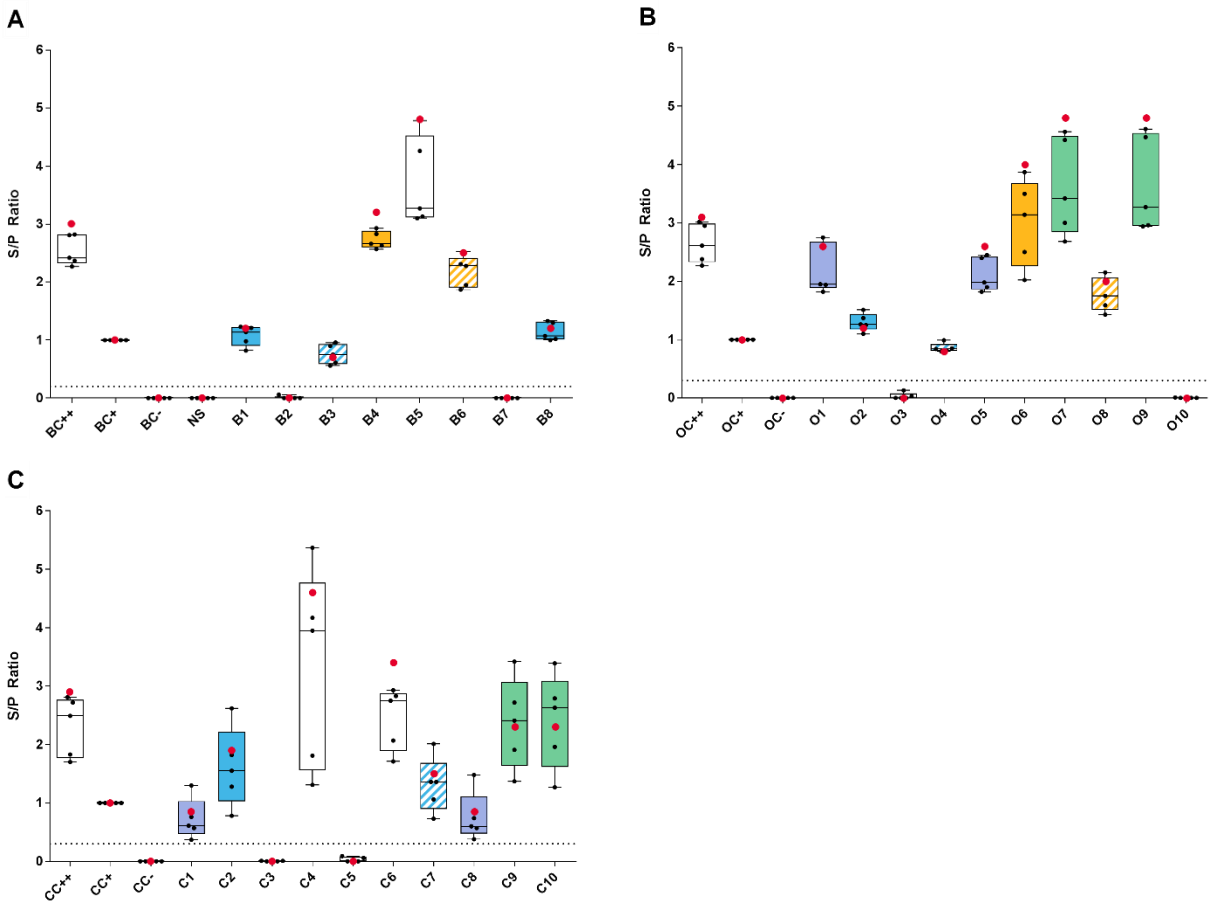


Fig. 1. Comparison of S/P ratios obtained by participating laboratories. S/P ratios for control and test sera are shown for cattle (Panel A), sheep (Panel B) and goat (Panel C) as box and whisker plots. In each panel individual data points are shown as black dots, the median values are shown by a horizontal line within each box, whiskers demarcate the minimum and maximum values, and the expected S/P ratio for each sample (based on data generated during homogeneity testing) is indicated by red dots. The assay cut-off value is depicted in each panel by a horizontal dashed line located at an S/P ratio of 0.2 for cattle sera, and 0.3 for sheep and goat sera. Within each panel identical pairs are identified by matched (solid) coloration of boxes, while split pairs are identified by matched coloration, but with the box colour of the more dilute sample patterned (striped) rather than solid in nature.

In addition, although the spread and variation in the data obtained was greater for the sheep and goat sera, as indicated by the summary statistics in Table 6, all laboratories correctly determined the order of the split pairs, with the higher numbered sample of each pair having the lower S/P ratio (Fig. 1).

Table 6. Summary statistics of test cattle (B1 to B8), sheep (O1 to O10) and goat (C1 to C10) sera derived from the S/P ratios obtained by the participating laboratories.
Abbreviations: IQR_N (normalised interquartile range); CV (coefficient of variation).

Sample	Median S/P ratio	IQR_N	Robust CV (%)	Minimum	Maximum	Range
B1	1.14	0.17	14.9	0.8	1.2	0.4
B2	0.00	0.00		0.0	0.1	0.1
B3	0.75	0.22	28.9	0.6	1.0	0.4
B4	2.66	0.15	5.5	2.6	2.9	0.4
B5	3.27	0.84	25.5	3.1	4.8	1.7
B6	2.28	0.27	11.7	1.9	2.5	0.7
B7	0.00	0.00		0.0	0.0	0.0
B8	1.07	0.20	19.1	1.0	1.3	0.3
O1	1.95	0.50	25.5	1.8	2.7	0.9
O2	1.26	0.09	7.5	1.1	1.5	0.4
O3	0.00	0.02		0.0	0.1	0.1
O4	0.84	0.02	2.8	0.8	1.0	0.2
O5	1.98	0.38	19.0	1.8	2.4	0.6
O6	3.14	0.74	23.6	2.0	3.9	1.9
O7	3.42	1.05	30.6	2.7	4.6	1.9
O8	1.75	0.29	16.4	1.4	2.1	0.7
O9	3.27	1.12	34.2	2.9	4.6	1.7
O10	0.00	0.00		0.0	0.0	0.0
C1	0.61	0.14	23.6	0.4	1.3	0.9
C2	1.55	0.41	26.2	0.8	2.6	1.8
C3	0.00	0.01		0.0	0.0	0.0
C4	3.95	1.75	44.2	1.3	5.4	4.1
C5	0.00	0.04		0.0	0.1	0.1
C6	2.75	0.56	20.5	1.7	2.9	1.2
C7	1.36	0.23	16.7	0.7	2.0	1.3
C8	0.60	0.13	21.0	0.4	1.5	1.1
C9	2.41	0.60	24.9	1.4	3.4	2.0
C10	2.63	0.61	23.4	1.3	3.4	2.1

4.5 Analysis of paired sera

The data for related sample pairs were analysed using Youden plots, as well as by generating Z-scores to facilitate the evaluation of both the between-laboratories and within-laboratory variation in the data generated by the five laboratories for which comparative data were available.

4.5.1 Cattle sera

Z-score analysis of the data for the identical sample pair 1 and 8 produced results for between-laboratory variation within normally accepted limits ($|Z| \leq 2.0$) for all laboratories (Fig. 2A). Although none of the Z-scores for within-laboratory variation denoted outliers, three labs (A, B and D) had intermediate Z-scores ($2.0 < |Z| < 3.0$) indicating there was greater variation in the paired data for these labs compared to the median of all the participating laboratories. The data points for these laboratories fell on or just outside the ellipse in either the upper left or lower right quadrants (Fig. 2B), suggesting that the results were influenced by random error components.

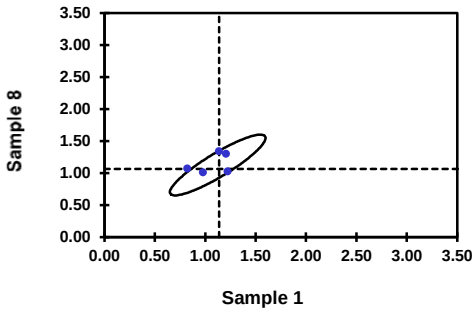
Z-score analysis of the data for both split sample pairs (samples 1 and 3; samples 4 and 6) produced results for between-laboratory and within-laboratory variation within the normally accepted limits ($|Z| \leq 2.0$) for all laboratories (Fig. 2C and Fig. 2E), with all data points falling within the 95% confidence region indicated by the ellipse (Fig. 2D and Fig. 2F).

Collectively, these data demonstrated acceptable reproducibility and repeatability for all three sample pairs.

A

Lab	S/P ratio		Between lab Z-score	Within lab Z-score
	Sample 1	Sample 8		
A	1.14	1.34	0.60	2.21
B	0.82	1.07	-0.95	2.71
C	1.21	1.30	0.68	0.99
D	1.23	1.02	0.00	2.29
E	0.98	1.00	-0.70	0.26

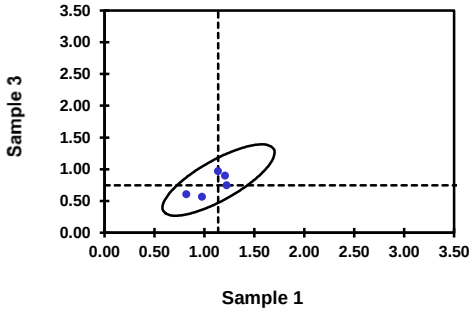
B



C

Lab	S/P ratio		Between lab Z-score	Within lab Z-score
	Sample 1	Sample 3		
A	1.14	0.96	0.30	-0.89
B	0.82	0.61	-1.26	-0.61
C	1.21	0.90	0.31	0.00
D	1.23	0.75	0.00	1.08
E	0.98	0.56	-0.99	0.69

D



E

Lab	S/P ratio		Between lab Z-score	Within lab Z-score
	Sample 4	Sample 6		
A	2.63	2.29	0.00	-1.06
B	2.94	2.31	1.02	0.00
C	2.66	2.53	0.87	-1.82
D	2.57	1.87	-1.46	0.24
E	2.83	1.95	-0.43	0.93

F

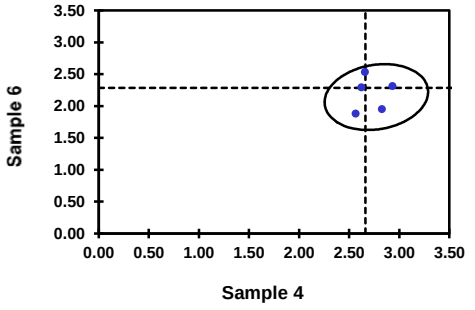


Fig. 2. Between and within laboratory analyses of related sample pairs (cattle sera). Z-scores and Youden plots are shown for identical sample pair 1 and 8 (Panels A and B), split sample pair 1 and 3 (Panels C and D) and split sample pair 4 and 6 (Panels E and F). S/P ratios of each laboratory's pair of results are plotted in blue, with an ellipse indicating the 95% confidence region (within which 95% of the results would be expected to lie in the absence of outliers in the data). The median value of each sample is illustrated by dashed lines perpendicular to the axes.

4.5.2 Sheep sera

Z-score analysis of the S/P ratios for identical sample pair 1 and 5 produced results for between-laboratory variation within the normally accepted limits ($|Z| \leq 2.0$) for all laboratories (Fig. 3A). Two labs (B and E) had intermediate within-laboratory Z-scores ($2.0 < |Z| < 3.0$) indicating there was greater variation in the paired data for these labs compared to the median of all the participating laboratories. The data points for these laboratories fell just on the ellipse in the upper right quadrant (Fig. 3B), suggesting that systematic error components influenced the results.

Z-score analysis of the data for the identical sample pair 7 and 9 produced similar results for between-laboratory variation (Fig. 3C). Analysis of the within-laboratory Z-scores identified laboratory D as being an outlier ($|Z| \geq 3.0$) and laboratory E as having an intermediate Z-score ($2.0 < |Z| < 3.0$), while the Z-scores for the remaining laboratories were within the normally accepted limits. The data points for labs D and E fell just outside the ellipse in either the lower left or upper right quadrants of the Youden plot (Fig. 3D). However, the higher within-laboratory Z-scores for these two labs are likely due to the greater variation in the paired data compared to those of the other participating laboratories, suggesting that the results were influenced by random error components.

Z-score analysis of the data for both split sample pairs (samples 2 and 4; samples 6 and 8) produced results for between-laboratory and within-laboratory variation within the normally accepted limits ($|Z| \leq 2.0$) for all laboratories (Fig. 3E and Fig. 3G), with the exception of laboratory D (samples 2 and 4), for which the between-laboratories Z-score was intermediate (Fig. 3E). The data points for all laboratories fell within the 95% confidence region indicated by the ellipse (Fig. 2F and Fig. 2H).

Overall, these data indicated acceptable reproducibility and repeatability for the two identical sample pairs and both split pairs.

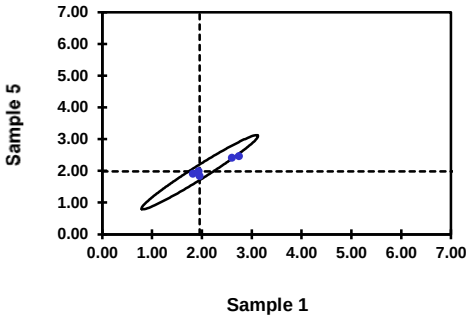
4.5.3 Goat sera

Z-score analysis of the data for the identical sample pair 1 and 8 produced results for between-laboratory variation within the normally accepted limits for all laboratories (Fig. 4A), except for laboratory C, which was identified as an outlier ($|Z| \geq 3.0$). The data points for this laboratory fell outside the ellipse in the upper right quadrant (Fig. 4B), while the data points for all other laboratories were within the ellipse, suggesting that systematic error components influenced the results. Analysis of the within-laboratory Z-scores identified laboratory A as having an intermediate Z-score ($2.0 < |Z| < 3.0$), while laboratory C was again identified as an outlier ($|Z| \geq 3.0$). However, this was likely due to the greater variation in the paired data compared to those of the other participating laboratories, suggesting that the results in this laboratory were also influenced by random error components.

A

Lab	S/P ratio		Between lab Z-score	Within lab Z-score
	Sample 1	Sample 5		
A	1.94	1.98	0.00	0.43
B	2.61	2.40	1.15	2.03
C	1.96	1.82	-0.15	1.28
D	1.82	1.90	-0.21	0.74
E	2.75	2.45	1.34	2.93

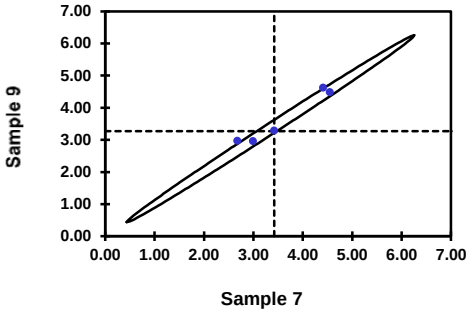
B



C

Lab	S/P ratio		Between lab Z-score	Within lab Z-score
	Sample 7	Sample 9		
A	3.00	2.94	-0.32	0.68
B	4.56	4.47	0.98	1.05
C	3.42	3.27	0.00	1.77
D	2.68	2.96	-0.44	3.31 §
E	4.42	4.61	0.98	2.35

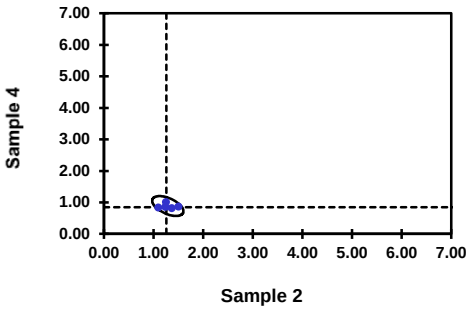
D



E

Lab	S/P ratio		Between lab Z-score	Within lab Z-score
	Sample 2	Sample 4		
A	1.26	0.99	0.62	-0.61
B	1.37	0.80	0.00	0.75
C	1.25	0.84	-0.68	0.00
D	1.10	0.82	-2.05	-0.54
E	1.51	0.85	1.49	1.15

F



G

Lab	S/P ratio		Between lab Z-score	Within lab Z-score
	Sample 6	Sample 8		
A	2.50	1.75	-0.85	-0.28
B	3.87	1.98	1.16	1.02
C	3.14	2.15	0.44	0.00
D	2.02	1.59	-1.66	-0.63
E	3.50	1.43	0.00	1.21

H

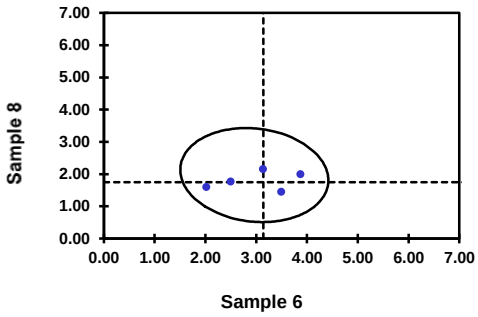
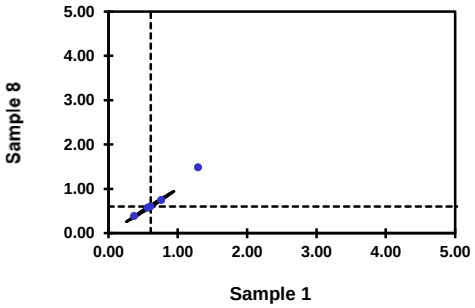


Fig. 3. Between and within laboratory analyses of related sample pairs (sheep sera). Z-scores and Youden plots are shown for identical sample pair 1 and 5 (Panels A and B), identical sample pair 7 and 9 (Panels C and D) split sample pair 2 and 4 (Panels E and F) and split sample pair 6 and 8 (Panels G and H). S/P ratios of each laboratory's pair of results are plotted in blue, with an ellipse indicating the 95% confidence region (within which 95% of the results would be expected to lie in the absence of outliers in the data). The median value of each sample is illustrated by dashed lines perpendicular to the axes. Note that a |Z-score| ≥ 3.0 indicates the result is an outlier (denoted by §).

A

Lab	S/P ratio		Between lab Z-score	Within lab Z-score
	Sample 1	Sample 8		
A	0.76	0.74	1.04	2.13
B	0.61	0.60	0.00	0.83
C	1.30	1.48	5.58 §	16.92 §
D	0.37	0.38	-1.62	0.83
E	0.57	0.57	-0.26	0.09

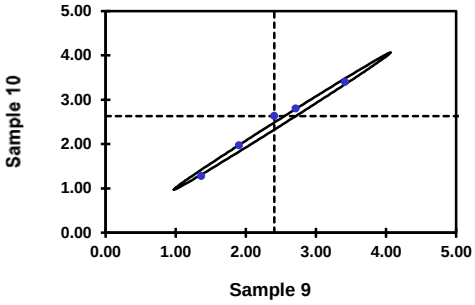
B



C

Lab	S/P ratio		Between lab Z-score	Within lab Z-score
	Sample 9	Sample 10		
A	1.91	1.96	-0.93	1.69
B	2.41	2.63	0.00	6.68 §
C	3.42	3.39	1.40	0.78
D	1.37	1.27	-1.90	2.98
E	2.72	2.79	0.37	2.26

D



E

Lab	S/P ratio		Between lab Z-score	Within lab Z-score
	Sample 2	Sample 7		
A	1.28	1.06	-0.88	0.00
B	1.83	1.36	0.41	1.15
C	2.62	2.01	2.62	1.82
D	0.78	0.73	-2.15	-0.76
E	1.55	1.36	0.00	-0.14

F

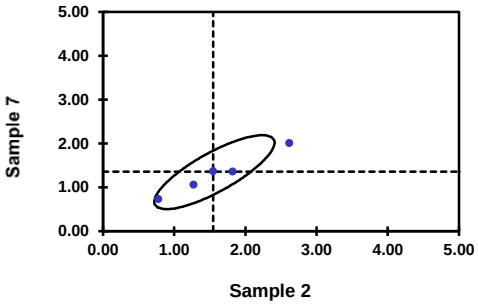


Fig. 4. Between and within laboratory analyses of related sample pairs. Z-scores and Youden plots are shown for identical sample pair 1 and 8 (Panels A and B), identical sample pair 9 and 10 (Panels C and D) and split sample pair 2 and 7 (Panels E and F). S/P ratios of each laboratory's pair of results are plotted in blue, with an ellipse indicating the 95% confidence region (within which 95% of the results would be expected to lie in the absence of outliers in the data). The median value of each sample is illustrated by dashed lines perpendicular to the axes. Note that a |Z-score| ≥ 3.0 indicates the result is an outlier (denoted by §).

Z-score analysis of the data for the identical sample pair 9 and 10 produced results for between-laboratory variation within the normally accepted limits for all laboratories (Fig. 4C). Analysis of the within-laboratory Z-scores identified laboratories D and E as having intermediate Z-scores ($2.0 < |Z| < 3.0$), and laboratory B as being an outlier ($|Z| \geq 3.0$). The data points for labs D and B fell just outside the ellipse in either the lower left or at the centre of all quadrants of the Youden plot, respectively (Fig. 4D). However, the higher within-laboratory Z-scores for these two labs are therefore likely due to the greater variation in the paired data compared to those of the other participating laboratories, suggesting that the results were influenced by random error components.

Z-score analysis of the data for the split sample pair 2 and 7 produced results for between-laboratory variation within the normally accepted limits for all laboratories (Fig. 4E), except C and D, which had intermediate Z-scores ($2.0 < |Z| < 3.0$). The data point for laboratory C was outside the ellipse in the upper right quadrant of the Youden plot (Fig. 4F), suggesting that the results were influenced by systematic error components. The within-laboratory variation was within the normally accepted limits ($|Z| \leq 2.0$) for all laboratories (Fig. 2E).

Together, these data indicated both acceptable reproducibility and repeatability for all three sample pairs.

5 Discussion

5.1 Comparability of results between and within the participating laboratories

Data for comparison were generated by five laboratories, which were located in five different countries on four continents. All five laboratories correctly classified all 8 cattle, all 10 sheep and all 10 goat sera as positive or negative following implementation of the test, representing 100% agreement based on qualitative interpretation of the normalised ELISA data. However, considerable variation was observed between the laboratories in the values obtained for the high and low positive control sera. Three laboratories obtained values within the expected range for the low positive cattle, sheep and goat sera, while only two obtained values within the expected range for the high positive cattle and sheep control sera, and three laboratories for the high positive control goat serum.

For laboratories where the OD values of the low positive control sera were outside the expected range, all but one exceeded the upper limit of the range. This meant that, in these laboratories, the ELISA substrate was developing beyond the linear dynamic range of the assay for the stronger positive samples. This resulted in a decrease in the S/P ratios relative to their expected values, since the developing substrate in the wells of the stronger positive test samples no longer increased at a constant rate in comparison to the wells containing the C+ control sera. Nevertheless, despite the suboptimal performance of the ELISA in several laboratories, this did not result in misclassification of any of the samples (either as false negative or false positive results).

Furthermore, based on the analysis of the blind coded identical and split paired sera that were included in each serum panel, the between-laboratory and within-laboratory Z-scores demonstrated acceptable reproducibility and repeatability were obtained. Of the ten paired sera tested, outlying results were observed for three pairs (one sheep and two goat) and, in each instance, by only one of the five participating laboratories. For all three pairs the outlying results related to within-laboratory testing of the sera, and were likely due to random errors, such as pipetting, influencing the repeatability of the assay for particular operators. For one of the paired goat sera the outlying result also related to between-laboratory testing and was more likely due to systematic error, since neither the ELISA plate readers nor batches of reagents were identical in every laboratory.

5.2 Batch-to-batch variability of the ELISA blocking / dilution buffer

As a greater number of data sets were returned for analysis, it became apparent that much greater variation than expected was evident in the results obtained for the control and test sera, particularly for the data generated using the goat samples. This prompted a review of the reagents and buffers that were specified for use by the participating laboratories. Three different batches of blocking buffer were evaluated at AAHL, in parallel (by the same operator), by testing the control cattle, sheep and goat sera while keeping all other ELISA reagents unchanged. Marked variation in the OD values of the high and low positive control sera was observed for each batch of blocking buffer used, increasing from ~0.5, for the low positive sera, to as high as ~0.8, ~1.1 and ~1.7 for cattle, sheep and goats, respectively (Fig. 5).

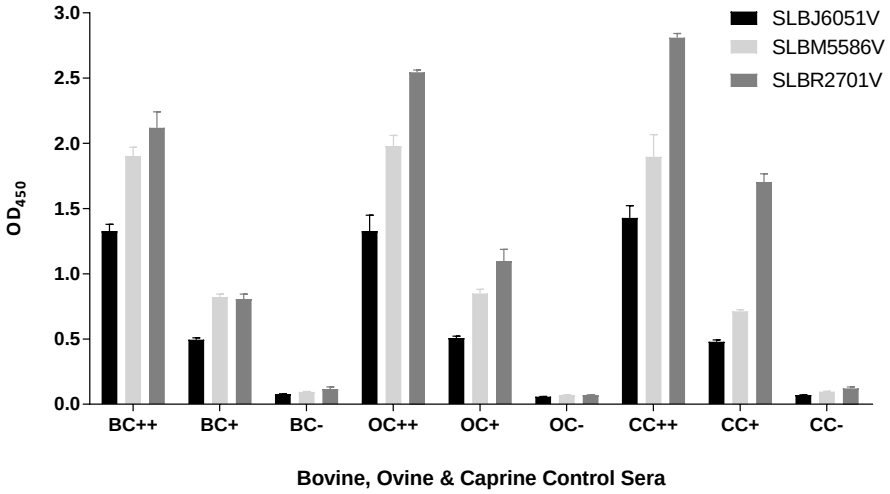


Fig. 5. Variation in OD values of cattle (B), sheep (O) and goat (C) high (C++), low (C+) and negative (C-) control sera observed when different batches of blocking buffer (Lot numbers SLBJ6051, SLBM5586V and SLBR2701V) were used. All other ELISA reagents were identical.

The variation in the performance of the assay was readily apparent in the OD values and S/P ratios obtained for the control sera when using each of the three different batches of blocking buffer (Table 7). For Lot # SLBJ6051V, all of the control cattle, sheep and goat sera were within the expected ranges. For Lot # SLBM5586V, the S/P ratios of the cattle and sheep high positive sera decreased to just below the lower limit of the expected range. For Lot # SLBR2701V, the OD value of the sheep low positive control serum was just outside the upper limit of the expected range, while it was well above for the goat low positive control serum. In contrast, the S/P ratios were below the lower limit of the expected range for the sheep and goat high positive control sera. Notably, these findings closely mirrored the sheep and goat control sera data obtained by laboratory D, which also used blocking buffer Lot # SLBR2701V (Table 7).

Considering that four different batches of blocking buffer were used by the five participating laboratories, it would therefore appear that the blocking buffer likely contributed to the between-laboratory variation observed in the results.

Table 7. Variation in OD values and S/P ratios obtained for control sera observed when different batches of blocking buffer were used. Ratios falling outside the expected range are indicated in bold.

Sample	Expected range	Blocking buffer lot number			
		SLBJ6051V	SLBM5586V	SLBR2701V	SLBR2701V (Lab D)
BC+	0.30 to 0.90	0.42	0.73	0.69	0.85
BC-	<0.20	0.08	0.10	0.12	0.08
BC++	2.6 to 3.4	3.0	**2.5**	2.9	**2.4**
OC+	0.30 to 0.90	0.45	0.78	**1.03**	**1.03**
OC-	<0.20	0.06	0.07	0.07	0.07
OC++	2.7 to 3.7	2.8	**2.5**	**2.4**	**2.4**
CC+	0.30 to 0.90	0.41	0.62	**1.58**	**1.98**
CC-	<0.20	0.07	0.10	0.12	0.08
CC++	2.5 to 3.4	3.3	2.9	**1.7**	**1.7**

5.3 Achievement of project objectives

The project objectives, and success in their achievement, are as follows:

1. *Confirm involvement of participating overseas laboratories (from Europe, North America or Africa) under appropriate agreements.*

   Achieved. In addition to the Diagnostic Emergency Response Laboratory, located at CSIRO AAHL in Geelong, seven overseas laboratories, from North America, Europe and Africa, participated in the inter-laboratory evaluation of the Capripox Cp 25 ELISA.

2. *Prepare and confirm reactivity of well-characterised control sheep, goat and cattle sera for distribution to overseas collaborating laboratories.*

   Achieved. Three separate panels of sera, comprising either bovine, ovine or caprine samples, were constructed using samples that had been previously characterised at NCFAD (Canada). The control sera comprised high and low positive, as well as negative, sera (C++, C+ and C-, respectively).

3. *Prepare and confirm reactivity of well-characterised test sheep, goat and cattle sera for distribution to overseas collaborating laboratories. Sera from sheep, goats or cattle would be included in each evaluation panel and would be blind coded prior to distribution.*

   Achieved. Three separate panels of blind coded sera, each comprising bovine, ovine or caprine samples, were chosen from samples that had been previously characterised at NCFAD (Canada). Reactivity of the sera was confirmed at NCFAD prior to shipment of the samples to AAHL, which required treatment with
   5 MRad gamma irradiation. Reactivity was again confirmed prior to panel preparation. The test sera included representative high, medium and low positive, as well as negative, sera. In addition, identical and split paired sera were included in each panel to facilitate assessment of both within and between laboratory variation of the assay.

4. *Distribute the control sera, evaluation panels, and all required ELISA materials and reagents to participating laboratories.*

Achieved. Following confirmation of the expected reactivity of all control and test sera, and testing of randomly selected aliquots of each control and test sample to confirm that they were sufficiently homogeneous, all consignments were shipped by air courier. All samples were confirmed by the participating laboratories to have arrived in good order, with samples arriving frozen on dry ice, except for one laboratory in Africa, for which the consignment took three months to clear customs.

5. *Coordinate the inter-laboratory evaluation of the ELISA using the sheep, goat and cattle serum panels.*

   Achieved. Data were received back from eight laboratories (located in Australia, North America, Europe and Africa).

6. *Collate and analyse data, and determine assay reproducibility.*

   Achieved. Useable data were received back from five of the eight laboratories, all of which correctly classified each test sample as positive or negative. Based on the analysis of the blind coded identical and split paired sera that were included in each serum panel, the between-laboratory and within-laboratory Z-score analyses demonstrated acceptable reproducibility and repeatability were obtained for all samples tested. Of the ten paired sera tested, outlying results were obtained for three sera and, in each instance, by only one of the five participating laboratories.

6 Conclusions/recommendations

The key outcome of this project has been the successful implementation of the Cp 25 ELISA in multiple overseas laboratories as part of an inter-laboratory evaluation of the assay. Additional validation data have therefore been generated which will facilitate adoption and eventual recognition of the assay internationally according to OIE guidelines (Stages 3 and 4 of the OIE Assay Development Pathway (Jacobson and Wright 2013)). This ELISA will address a major deficiency in the capability and preparedness of AAHL, as well as other laboratories internationally, for undertaking effective post-outbreak sero-surveillance for sheeppox, goatpox, and lumpy skin disease, which would be critical for re-establishing disease free status and expediting the resumption of trade in livestock and livestock products in the event of an outbreak.

Of the five laboratories that returned useable data, all correctly classified the cattle, sheep and goat sera as positive or negative following implementation of the test, representing 100% agreement based on qualitative interpretation of the normalised ELISA data. However, considerable variation was observed between the laboratories in the values obtained for the high and low positive control sera. Nevertheless, despite the suboptimal performance of the ELISA in several laboratories, this did not result in misclassification of any of the test samples. Furthermore, based on the analysis of the blind coded paired sera that were included in each serum panel, the between-laboratory and within-laboratory Z-score analyses confirmed acceptable reproducibility and repeatability for all samples tested. Outlying results were obtained for only three paired sera and, in each instance, by only one of the five participating laboratories.

Subsequent review of the available commercial reagents and buffers that were specified for use in the ELISA protocol identified the commercial blocking buffer as a likely contributor to the between-laboratory variation observed in the results. This particular buffer will need to be replaced to improve the robustness of the assay prior to implementation internationally, and this would be best undertaken by conversion of the ELISA into kit form in collaboration with a commercial partner.

Commercialisation of the Cp 25 ELISA will:

- Facilitate international acceptance of the assay as a new OIE listed test;
- Make the assay available in kit form, with quality controlled reagents and pre-coated plates standardised to “ELISA quality”;
- Improve quantitative repeatability and reproducibility of the assay;
- Allow widespread availability internationally for investigation of potential outbreaks, as well as for ongoing testing during control and eradication programs; and
- Enhance Australia's capability to counter an incursion of disease (sheeppox, goatpox or lumpy skin disease) caused by capripoxviruses.

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9 Appendices

9.1 Appendix 1 - Instructions for inter-laboratory evaluation using cattle, sheep and goat serum panels

Test procedure

Day 1 (Afternoon)

1. Coat a NUNC MaxiSorp ELISA plate with recombinant protein Cp25. Dilute 6 µl of Cp25 in 10 ml of 0.05 M carbonate-bicarbonate coating buffer (per plate). Mix well (invert gently 10 times) to ensure homogeneity and add 100 µl to each well of the ELISA plate using a multichannel pipette.

2. Cover with plate sealer and incubate overnight at 4°C in a sealed box (inner base lined with paper towel moistened with water). Plates should not be stored longer than overnight and must be used the following day.

Day 2

N.B. Allow all diluents / buffers to reach room temperature (20-25°C) before addition to the plate.

3. Wash plate 4 times with PBST using plate washer, invert plate and tap on absorbent paper towel to remove residual liquid from wells. Plate should not be allowed to dry out before proceeding with the next step.

   Alternatively, a squeeze bottle may be used to wash the plate by hand. Fill each well with 300 µl of PBST, stand 5 seconds per rinse, empty the wells completely between rinses and, following the final rinse, invert the plate and tap on absorbent paper towel to remove excess liquid. Plate should not be allowed to dry out before proceeding with the next step.

4. Block plate by adding 200 µl per well of 1X Casein blocking buffer.

5. Cover with plate sealer and incubate for 60 minutes at 37°C on plate shaker.

6. While plate is blocking, dilute control and test sera 1:100 in 1X Casein blocking buffer in a Nunc Microwell transfer plate (pipette 2.5 µl of serum into 247.5 µl of 1X Casein blocking buffer) as per the template in Fig. 1 (Appendix 1). Change pipette tips between each and every well.

7. After plate has finished blocking, wash plate 4 times with PBST (as for Step 3).

8. Using a multichannel pipette, transfer diluted sera to the ELISA plate one column at a time. Ensure complete mixing of sera and diluent in each column, by pipetting up and down 8 times, prior to transfer. Duplicate 100 µl aliquots of each mixed sample are transferred to neighbouring columns in the ELISA plate according to the template in Fig. 2 (Appendix 1). For each column of the transfer plate the same pipette tips can be used for mixing and transfer of samples, in duplicate, to the ELISA plate, but tips must be changed when moving to the next column of the transfer plate.

9. Cover ELISA plate with plate sealer and incubate for 60 minutes at 37°C on plate shaker.
10. Wash plate 4 times with PBST (as for Step 3).
11. Dilute Protein G-HRP conjugate 1:128,000 in 1X Casein blocking buffer and add 100 µl per well.
12. Cover with plate sealer and incubate for 60 minutes at 37°C on plate shaker.
13. Dispense 12 ml of TMB substrate into a clean plastic tube, protect from light (wrap in foil or place in a closed drawer or cupboard), and allow to reach room temperature (20-25°C) before use.
14. At the end of the conjugate incubation, wash plate 4 times with PBST (as for Step 3).
15. Add 100 µl per well of TMB substrate to all wells and incubate the plate, stationary on the bench (do not shake or mix), at room temperature (20-25°C) for 10 minutes. Add TMB in a predetermined manner, for example from Row A to H, and start timing after the first row is filled.
16. Stop substrate reaction by the addition of 100 µl per well of STOP solution (1 M H_2SO_4). Add STOP solution in the same manner as TMB so that all wells have the same incubation time.
17. Ensure the plate reader has warmed up for the required time as specified in the user manual. Read raw Optical Density values at 450 nm without blanking against any wells. Ensure there are no air bubbles in the wells and gently clean the underside of the ELISA plate with lint free tissue prior to reading (plates should be read immediately or within 10 minutes from addition of STOP solution).

Data Transformation and Interpretation

For each species (bovine, ovine and caprine), mean OD values are calculated for the control and sample wells. These values are then corrected by subtracting the mean OD value of the negative control reference serum of each species (BC-, OC-, CC-) from the mean OD value of the respective control and test samples. Results are expressed as a ratio of the corrected mean OD value of the test sample (S) to the corrected mean OD value of the low positive control (P) reference serum (BC+, OC+ or CC+, respectively).

$$S/P = \frac{(Mean\ OD\ of\ Sample) - (Mean\ OD\ of\ C-)}{(Mean\ OD\ of\ C+) - (Mean\ OD\ of\ C-)}$$

These calculations, and those pertaining to the controls below, are performed automatically when the raw OD values are copied and pasted into the Excel data template provided.

Based on current validation data the test is deemed valid if the following criteria are met.

Bovine control sera

Mean OD BC(+) - BC(-)	0.30 to 0.90
Mean OD BC(-)	<0.20
S/P BC(++)	2.6 to 3.4

Samples with an S/P ratio <0.2 are considered negative.

Samples with an S/P ratio ≥0.2 are considered positive. Duplicate wells of positive sera should have OD values that differ from the mean by no more than 15%.

Ovine control sera

Mean OD OC(+) - OC(-)	0.30 to 0.90
Mean OD OC(-)	<0.20
S/P OC(++)	2.7 to 3.7

Samples with an S/P ratio <0.3 are considered negative.

Samples with an S/P ratio ≥0.3 are considered positive. Duplicate wells of positive sera should have OD values that differ from the mean by no more than 15%.

Caprine control sera

Mean OD CC(+) - CC(-)	0.30 to 0.90
Mean OD CC(-)	<0.20
S/P CC(++)	2.5 to 3.4

Samples with an S/P ratio <0.3 are considered negative.

Samples with an S/P ratio ≥0.3 are considered positive. Duplicate wells of positive sera should have OD values that differ from the mean by no more than 15%.

ELISA Summary procedure

1. Coat a NUNC MaxiSorp ELISA plate with 100 µl per well of recombinant protein Cp25 (6 µl of Cp25 diluted in 10 ml of 0.05 M carbonate-bicarbonate coating buffer).
2. Cover with plate sealer and incubate overnight at 4°C in a sealed box (inner base lined with paper towel moistened with water).
3. Wash plate 4x with PBST using plate washer (or by hand using squeeze bottle).
4. Block plate by adding 200 µl per well of 1X Casein blocking buffer.
5. Cover with plate sealer and incubate for 60 min at 37°C on plate shaker.
6. Dilute control and test sera 1:100 in blocking buffer (pipette 2.5 µl of serum into 247.5 µl of 1X Casein blocking buffer) in Nunc Microwell transfer plate according to the template in Fig. 1 (Appendix 1).
7. After plate has finished blocking, wash plate 4x with PBST using plate washer (or by hand using squeeze bottle).
8. Using a multichannel pipette mix and transfer 100 µl of the diluted sera from the transfer plate to the ELISA plate, one column at a time, so that each sample is duplicated in neighbouring columns in the ELISA plate (Fig. 2, Appendix 1).
9. Cover with plate sealer and incubate for 60 min at 37°C on plate shaker.
10. Wash plate 4x with PBST using plate washer (or by hand using squeeze bottle).
11. Dilute Protein G-HRP conjugate 1:128,000 in 1X Casein blocking buffer and add 100 µl per well.
12. Cover with plate sealer and incubate for 60 min at 37°C on plate shaker.
13. Wash plate 4x with PBST using plate washer (or by hand using squeeze bottle).
14. Add 100 µl per well of TMB substrate to all wells and incubate at room temperature for 10 min.
15. Stop substrate reaction by the addition of 100 µl per well of STOP solution (1 M H_2SO_4).
16. Read raw Optical Density values at 450 nm on plate reader.
17. Copy and paste the raw OD values into Section 1 of the Excel data template provided for automatic calculation of S/P ratios.

Transfer Plate and ELISA Plate Templates

	1	2	3	4	5	6	7	8	9	10	11	12
A	BC++	B1	OC++	O3	CC++	C3						
B	BC++	B2	OC++	O4	CC++	C4						
C	BC+	B3	OC+	O5	CC+	C5						
D	BC+	B4	OC+	O6	CC+	C6						
E	BC-	B5	OC-	O7	CC-	C7						
F	BC-	B6	OC-	O8	CC-	C8						
G	NS	B7	O1	O9	C1	C9						
H	NS	B8	O2	O10	C2	C10						

Fig. 1. Template for dilution of control and test sera in a Nunc Microwell transfer plate. 1X Casein blocking buffer is added to columns 1 to 6 (247.5 µl per well). Control and test sera (2.5 µl per well) are then added sequentially, column by column, starting at well A1 (change tips between every well; no need to mix sera at this stage). Abbreviations: BC, Bovine control sample; NS, no serum (diluent only (1X Casein blocking buffer)); OC, Ovine control sample; CC, Caprine control sample; B1 to B8, Bovine test samples 1 to 8; O1 to O10, Ovine test samples 1 to 10; and C1 to C8, Caprine test samples 1 to 10.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BC++	BC++	B1	B1	OC++	OC++	O3	O3	CC++	CC++	C3	C3
B	BC++	BC++	B2	B2	OC++	OC++	O4	O4	CC++	CC++	C4	C4
C	BC+	BC+	B3	B3	OC+	OC+	O5	O5	CC+	CC+	C5	C5
D	BC+	BC+	B4	B4	OC+	OC+	O6	O6	CC+	CC+	C6	C6
E	BC-	BC-	B5	B5	OC-	OC-	O7	O7	CC-	CC-	C7	C7
F	BC-	BC-	B6	B6	OC-	OC-	O8	O8	CC-	CC-	C8	C8
G	NS	NS	B7	B7	O1	O1	O9	O9	C1	C1	C9	C9
H	NS	NS	B8	B8	O2	O2	O10	O10	C2	C2	C10	C10

Fig. 2. Template for location of diluted control and test sera following transfer to the Nunc MaxiSorp ELISA plate coated with Cp25. Samples are transferred sequentially from Column 1 to 12, using a multichannel pipette, with Columns 1 and 2 containing Bovine control sera (in quadruplicate), Columns 3 and 4 Bovine test sera (in duplicate), Columns 5 and 6 Ovine control (in quadruplicate) and test (in duplicate) sera, Columns 7 and 8 Ovine test sera (in duplicate), Columns 9 and 10 Caprine control (in quadruplicate) and test (in duplicate) sera, and Columns 11 and 12 Caprine test sera (in duplicate). i.e. samples from Column 1 of the transfer plate are transferred to columns 1 and 2 of the ELISA plate (using the same pipette tips); using new tips samples from column 2 of the transfer plate are transferred to columns 3 and 4 of the ELISA plate, etc. Abbreviations: BC, Bovine control sample; NS, no serum (diluent only (1X Casein blocking buffer)); OC, Ovine control sample; CC, Caprine control sample; B1 to B8, Bovine test samples 1 to 8; O1 to O10, Ovine test samples 1 to 10; and C1 to C8, Caprine test samples 1 to 10.