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Development and evaluation of a rapid immunomagnetic bead assay for the detection of classical swine fever virus antigen

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Abstract

Classical swine fever (CSF) is a highly contagious and severe viral disease of swine resulting in substantial production losses in different farming systems in many regions of the world. The accurate and rapid detection of CSF outbreaks is reliant on sensitive and specific laboratory testing and is a key component of disease control. Specific detection of CSF virus can be achieved by virus isolation in tissue culture, antigen capture or the detection of viral

RNA using molecular techniques. In order to reduce the time taken to achieve a diagnostic result and simplify testing methods, an antigen capture ELISA using immunomagnetic beads (IMB) as the solid phase was developed and compared to a microplate-based antigen capture (AC)-ELISA. The IMB-ELISA has up to 64-fold greater analytical sensitivity than the AC-ELISA and initial estimates of diagnostic sensitivity and specificity are 100%. The IMB-ELISA has a highly robust, rapid and stable test format and is simpler to perform than the AC-ELISA. The IMB-ELISA has the added advantage that a result can be sensitively and specifically determined by eye, lending it to the possibility of adaptation to a near-to-field test with minimal equipment or expertise needed.

Keywords: Classical swine fever virus; Diagnosis; Rapid test; Immunomagnetic; Bead; Microparticle; ELISA

Introduction

Classical swine fever (CSF) virus is a member of the pestivirus genus within the family Flaviviridae (Heinz et al. *2000*; Pringle *1999*). CSF is a highly contagious and pathogenic disease having severe impacts on pig production systems. A definitive diagnosis of CSF is reliant on accurate laboratory testing owing to the difficulty of making a diagnosis based solely on clinical signs (Elbers et al. *2002*, *2004*; Paton and Greiser-Wilke *2003*; Van Oirschot *1999*). Laboratory diagnostic methods include isolation and detection of virus in tissue culture, detection of viral antigen, the demonstration of a rise in the titre of virus-induced antibody or the detection of specific viral RNA. Specific diagnosis requires a test to

be able to discriminate between CSF virus and other closely related pestiviruses such as bovine viral diarrhoea (BVD) virus and border disease (BD) virus.

At present, all methodologies are reliant on having at least rudimentary laboratory facilities (Paton and Greiser-Wilke *2003*) and experienced operators. The choice of diagnostic test used in a particular laboratory is dependent on the local setting, resources available and the climatic conditions in which samples are collected and transported. This is particularly important in tropical countries with limited infrastructure; delays in sample transport to the laboratory may be common and ambient tropical temperatures detrimental to sophisticated methods such as RT-PCR (Blacksell et al. *2004b*) and virus isolation.

This study describes the development and evaluation of an antigen capture ELISA in the limited resource setting of the Lao People's Democratic Republic (Lao PDR). Immunomagnetic beads (IMB) were used as the solid phase to improve the speed and simplicity of CSF diagnosis without compromising sensitivity, specificity and robustness.

Materials and methods

Clinical samples

One hundred and fifty eight spleen specimens from CSF-suspected pigs were submitted to the National Animal Health Centre, Vientiane from 2002 to 2004. Specimens were transported in buffered glycerol (50% v/v glycerol in phosphate buffered saline (PBS)), prepared as 20%

w/v spleen homogenates in buffered detergent (1% v/v Nonidet P-40 in PBS) and stored at −85°C prior to testing.

Control antigens

Spleen tissue was homogenised in buffered detergent followed by centrifugation at 2000 rpm for ten minutes at 25°C. The CSF positive antigen extract was prepared as a pooled 5% w/v spleen homogenate from three indigenous black pigs infected with a local strain in Vientiane, Lao PDR. The negative antigen extract was prepared in the same manner using six spleens collected from non-indigenous white pigs in Vientiane that were confirmed CSF viral antigen negative by AC-ELISA. No animals were used experimentally during the course of this study.

Antibodies

Anti-pestivirus goat polyclonal capture antibody was produced and supplied by the CSIRO Australian Animal Health Laboratory (AAHL) (Geelong, Australia). The hyperimmune goat serum was purified by caprylic acid/ammonium sulphate precipitation (McKinney and Parkinson *1987*) and reconstituted in PBS.

Monoclonal antibody (MAb) 24/10 specific for the E2 protein of CSF virus was produced at CSIRO AAHL and used as tissue culture supernatant without purification. The MAb was first produced at the Federal Research Centre for Virus Diseases of Animals, Tubingen, Germany

using the Thiverval vaccine strain (Weiland et al. *1990*) and was further characterised by Kosmidou et al. (*1995*).

Horse-radish-peroxidase conjugated to goat anti-mouse IgG was supplied by Dako Cytomation, Denmark.

Coating of IMBs with capture antibody

Carboxyl modified polystyrene paramagnetic microparticles (Spherotech Inc., USA) (1% w/v) were combined with polyclonal capture antibody and coated in the presence of 1 mM 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma Aldrich, USA). The microparticles were separated on a 1.5S LifeSep magnet (Dexter Magnetic Technologies, USA) and subsequently blocked (2% w/v fish skin gelatine {Sigma Aldrich, USA}, 0.07% v/v Proclin 300 {Sigma Aldrich, USA} in PBS) and stored at a final concentration of 0.5% w/v in storage buffer (0.5% w/v fish skin gelatine, 0.07% v/v Proclin 300 in PBS). The polyclonal antibody coated microparticles are henceforth referred to as immunomagnetic beads (IMB).

IMB-ELISA method

Twenty five microliters of coated IMBs were added in duplicate to samples and controls. The tubes were vortexed and incubated at 37°C for 30 minutes with gentle agitation to prevent settling of beads. The tubes were placed on a magnet for at least 20 seconds and the supernatant discarded. The IMBs were washed with 0.05% Tween 20 PBS followed by the

addition of MAb 24/10 diluted in diluent buffer (5% v/v glycerol, 0.5% w/v fish skin gelatine, 0.05% v/v Tween 20, 0.07% v/v Proclin 300 in PBS). The tubes were vortexed and agitated for 30 minutes at 37°C followed by washing. Conjugate diluted in diluent buffer was then added, mixed and agitated for 30 minutes at 37°C. The IMBs were washed three times and transferred to a new tube followed by the addition of chromogen-substrate (0.42 mM 5′, 5′-tetramethylbenzidine {Sigma Aldrich, USA}, 0.004% v/v H_2O_2 in 0.1 M acetate buffer, pH 5.9), incubated at 25°C for 5 minutes and the reaction stopped with 1N H_2SO_4. The supernatant was transferred to a flat bottomed 96-well microplate (Nunc, Denmark) and the optical density (OD) measured at 450 nm. The results were recorded as percent positivity of the CSF positive control according to the equation:

$$\%\,\text{Positivity} = \frac{\text{Sample OD}_{450\text{nm}} - \text{Negative Control OD}_{450\text{nm}}}{\text{Positive Control OD}_{450\text{nm}} - \text{Negative Control OD}_{450\text{nm}}} \times 100$$

Antigen capture-ELISA for CSF virus

The antigen capture-ELISA (AC-ELISA) used in this study was developed at CSIRO AAHL based on that described by Shannon et al. (*1993*) with local modifications (Fuqing et al. *2000*). The test relies on the use of three monoclonal antibodies to infer the presence of CSF virus antigen: (1) pestivirus group reactive; (2) bovine viral diarrhoea virus-specific; and (3) pestivirus negative antibodies. The results of the test were expressed as a signal-to-noise (S/N) ratio, where an S/N ratio >2 was indicative of the presence of CSF virus antigen (Fuqing et al. *2000*; Shannon et al. *1993*).

IMB-ELISA cut-off

Archived specimen preparations were diluted 1:4 in sample dilution buffer (10% v/v normal goat serum, 0.05% v/v Tween 20, 0.07% v/v Proclin 300 in PBS) to a total volume of 100 µL and left at 25°C for 20 minutes. The IMB-ELISA was performed as described above. A scatter-plot of the results obtained for the AC-ELISA and IMB-ELISA was constructed to estimate diagnostic cut-off combined with modified receiver-operator-curve (ROC) analysis (Jacobson *1998*) to justify the selected cut-off. Correlation was calculated by Spearman's rank test using Stata/SE 8.0 software (Stata Corp., College Station, Texas).

Validation and evaluation of IMB-ELISA

Repeatability

Aliquots of CSF positive and negative control antigens were stored at − 85°C before testing in the assay. Four replicates of each control were tested in the IMB-ELISA in each of 15 assays, where each assay used a different batch of coated IMBs. The mean, standard deviation and coefficient of variation were calculated for each set of replicates to determine intra-batch and inter-batch variation.

Stability of IMB-antibody complex

Four replicates of each control antigen were tested in the IMB-ELISA immediately after preparing a batch of coated IMBs and again at week one, two, four, six, eight and ten. The coated IMBs were stored at 4°C throughout. Monoclonal antibody 24/10 and conjugate were

diluted in diluent buffer on the day of the test and chromogen-substrate was made immediately before use.

Analytical sensitivity

The analytical sensitivity of the IMB-ELISA and the AC-ELISA were determined using spleen, tonsil and kidney tissue from two diagnostic submissions collected in 2004: sample numbers 089/04 and 091/04. Tissue was transported in PBS buffered glycerol and both were received at the laboratory within 8 hours of collection. The titre of virus in each specimen was determined by titration in tissue culture using methods similar to those described elsewhere (Kamolsiriprichaiporn et al. *1992*) and stained by peroxidase linked assay (Jensen *1981*). Homogenates (5% w/v) of spleen, tonsil and kidney were prepared in buffered detergent and serially two-fold diluted and tested in the IMB-ELISA and AC-ELISA. Positive and negative controls were included in each test to calculate the percent positivity for the IMB-ELISA and S/N ratio for the AC-ELISA.

Inter-operator agreement

The level of inter-operator agreement for the IMB-ELISA was assessed using three operators, two experienced and one with limited laboratory experience. Forty samples (25%) were selected from the 158 field specimens comprising 20 randomly selected negatives (according to the cut-off), 11 samples just above or below the diagnostic cut-off and nine randomly selected strong positives. Samples were prepared in duplicate and stored at − 85°C in 100 µL aliquots. Each operator assessed the same samples independently and each sample was assigned a unique identification number for each operator to prevent comparison of scores.

All operators prepared their own reagents and the IMB-ELISA was performed with the inclusion of duplicate positive and negative controls. Before the chromogen-substrate reaction was stopped, each operator scored samples as positive for a blue/green colour and negative for a brown colour. The reaction was then stopped, the supernatant transferred to an ELISA plate, the OD measured at 450 nm and the percent positivity was calculated relative to the strong positive control. The inter- and intra-agreement of operators was assessed by kappa statistic analysis (Smith *2006*) where 0.41–0.60, 0.61–0.80, 0.81–0.99 and 1.00 kappa scores correspond to moderate, substantial, almost perfect and perfect agreement, respectively. The diagnostic sensitivity and specificity of each operator's results were calculated using EpiCalc software (CDC, USA) with the AC-ELISA result for each sample used as the reference comparator.

Results

Determination of diagnostic cut-off

Of the 158 field specimens tested, 39 (25%) were positive by AC-ELISA. The test results for both the IMB- and AC-ELISA were not normally distributed and using the non-parametric Spearman's rank test, a significant correlation between the two tests was demonstrated (rho = 0.705, $p < 0.00005$). At a cut-off of 10% positivity in the IMB-ELISA, the relative diagnostic sensitivity and specificity were both 100% (95% CI: 89–100% and 96–100%, respectively). At a cut-off of 5% positivity, the relative diagnostic sensitivity and specificity were 100% and 88% respectively (95% CI: 89–100% and 89–98%, respectively) (Fig. 1). On the basis of these results it was decided to use a cut-off of equal to or greater than 10% positivity.

Repeatability

The mean corrected OD for four replicates over 15 runs (60 replicates) was 1.64 (sd = 0.14; cv = 8.75). Ten of the 15 runs (67%) resulted in an OD within one standard deviation of the overall mean; the remaining five (33%) were within two standard deviations of the mean. The median coefficient of variation of each of the 15 runs was 3.48 (range: 1.21 to 7.23). The mean OD of the negative control for the four replicates over 15 runs (60 replicates) was 0.07 (sd = 0.03; range: 0.03 to 0.14).

Stability of IMB - antibody complex

The IMB bound capture antibody remains stable for at least 10 weeks, with no observed difference between the OD at week 0 and week 10.

Analytical sensitivity

The analytical sensitivity of the IMB-ELISA was demonstrated to be greater than that of the AC-ELISA (Table 1). The limit of detection of viral antigen in spleen tissue was 32-fold greater in the IMB-ELISA compared to the AC-ELISA. In the case of tonsil tissue, the AC-ELISA was able to positively detect CSF virus antigen in only one specimen (089/04); whereas the IMB-ELISA was able to detect antigen in both specimens with a greater than 64-fold increase in analytical sensitivity. Viral antigen from infected kidney tissue could only be detected by IMB-ELISA in sample 089/04. Both ELISA procedures were able to detect fewer units of infectious virus in tonsil tissue as compared to spleen and kidney tissue. An approximately 10-fold increase in analytical sensitivity for tonsil tissue was observed in comparison to spleen and kidney tissue when tested by the IMB-ELISA. For specimen

089/04, a 5-fold increase in analytical sensitivity was observed in tonsil tissue compared to spleen tissue when tested by the AC-ELISA.

Inter-operator agreement

When results were determined by calculating percent positivity (positive samples ≥10% of the strong positive control), agreement between Operators 1 and 2 was almost perfect ($k_{1,2} = 0.90$) and agreement between Operators 1 and 3 and Operators 2 and 3 were substantial ($k_{1,3} = 0.74$; $k_{2,3} = 0.74$). When samples close to the diagnostic cut-off were assessed, variation above and below the 10% cut-off was observed between the operators (Fig. 2). Similar results were observed when the test result was read by eye; agreement between Operators 1 and 2 was almost perfect ($k_{1,2} = 0.95$) and between Operators 1 and 3 and Operators 2 and 3 the agreement was substantial ($k_{1,3} = 0.80$; $k_{2,3} = 0.75$). The level of agreement of reading a result by eye or calculating percent positivity was determined for each operator. The levels of agreement for Operators 1 and 2 were almost perfect, ($k_1 = 0.95$; $k_2 = 0.90$) and Operator 3 had substantial agreement between reading by eye and calculating percent positivity ($k_3 = 0.79$). The relative diagnostic sensitivity and specificity were also determined for each operator's results using the AC-ELISA as the reference comparator. Whether the result was determined by eye or by percent positivity, the relative diagnostic sensitivity remained consistent. This was particularly the case for Operators 1 and 2 (Table 2) with a corresponding negative predictive value of one, demonstrating a confidence that all negative results are truly negative. It appears from the small number of samples tested that the relative diagnostic specificity suffered slightly when the IMB-ELISA was read by eye.

Discussion

This study has described the development and validation of a method for the detection of CSF virus antigen that uses immunomagnetic bead technology and is simple, inexpensive and suited to field application. The IMB-based test for the detection of CSF virus was sensitive and specific using a polyclonal antibody with pestivirus group reactivity in combination with a species specific monoclonal antibody. Kosmidou et al. (*1995*) demonstrated that the monoclonal antibody used in this test (clone 24/10, panel number 7) was reactive with a wide range of CSF virus reference and vaccine strains and non-reactive with a panel of BVD virus reference strains. Only subgroup 2.1 and 2.2 viruses have been detected in the Lao PDR (Blacksell et al. *2004a*, *2005*) and the IMB-ELISA was able to detect all Lao isolates tested during this study. The test was also able to sensitively detect the Weybridge CSF virus strain (data not shown) belonging to subgroup 1.1 (Tu et al. *2001*). Given the high level of antigenic conservation, it is likely that CSF virus belonging to other genotypes will react in this IMB-ELISA. However, to conclusively demonstrate the accuracy of this assay, additional CSF virus reference strains and a panel of BVD and BD viruses need to be tested.

Virus isolation has been considered the diagnostic gold standard for CSF (de Smit *2000*; Pearson *1992*), however recent studies have demonstrated that real-time RT-PCR has greater diagnostic sensitivity (Hoffmann et al. *2005*; Risatti et al. *2005*). In this study, the AC-ELISA was used as the reference standard to determine the diagnostic cut-off of the IMB-ELISA because it was the most suited test for the Lao setting. Prolonged specimen transport times (beyond three days) at tropical ambient temperatures result in false negatives in the RT-PCR (Blacksell et al. *2004b*), and virus isolation from degraded specimens was impractical. While it is recognised that the AC-ELISA was not the most sensitive diagnostic comparator to

determine the diagnostic cut-off, the AC-ELISA was the most appropriate reference test for the conditions under which the IMB assay was developed and will be used.

All diagnostic specimens positive by AC-ELISA were also positive in the IMB-ELISA at a cut-off of greater than or equal to ten percent positivity, likewise for negative specimens. Even though there was a large variation in AC-ELISA S/N ratio values for specimens with a percent positivity greater than 80, a significant linear correlation between the two tests was demonstrated. This was expected however, because both tests use a similar principle of antigen capture and subsequent detection by monoclonal antibody.

The IMB-ELISA was shown to be a highly repeatable and stable assay, however, results approaching the cut-off can fluctuate above and below depending on the operator. As a consequence, the diagnostic performance of the IMB assay may not always be equivalent to the AC-ELISA. But the variability of a small proportion of specimens is not likely to strongly influence diagnostic sensitivity and specificity. A blinded trial will be necessary to determine a true measure of diagnostic performance and the influence of inter-operator agreement on these results further investigated. A test with diagnostic sensitivity and specificity greater than 90% will be more than sufficient to identify outbreaks of CSF, especially in an endemic environment and when more than one specimen is tested from any suspected outbreak of disease.

The IMB-ELISA was at least 32 to 64-fold more sensitive than the AC-ELISA for the detection of CSF virus antigen and will be of advantage for the detection of low virulent

strains that grow to lower titres in organs and in settings where sample degradation is a problem. This result compares favourably with virus isolation, which was found to be in the order of 10- to 100-fold more sensitive than AC-ELISA (McGoldrick et al. *1999*).

Technician or operator error is the highest potential source of variation for many assays and the level of agreement between operators is a measure of the relative repeatability of an assay (Jacobson *1998*). Determining a test result by observing a colour change is subjective. The agreement between the operator's results of this study was very high, whether read by eye or determined by measuring optical density. The level of agreement presented from the IMB-ELISA demonstrated between-operator consistency making the test suitable for application in a low-technology setting where access to ELISA plate readers and other specialist equipment is limited or non-existent.

Currently there are no rapid tests for the field, or near-to-field, diagnosis of CSF. The IMB-ELISA is simple to perform, rapid, cheap and results can be read reliably by eye with minimal training required. Future field-based studies are required to prove the utility of the IMB-ELISA for disease control purposes in limited-resource settings.

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Fig. 1. (A) IMB-ELISA verses AC-ELISA and (B) Modified ROC plot of the diagnostic sensitivity (solid black line) and specificity (dashed line) of the IMB-ELISA as a function of cut-off intervals (x-axis). Diagnostic sensitivity and specificity were measured against the AC-ELISA as a reference standard

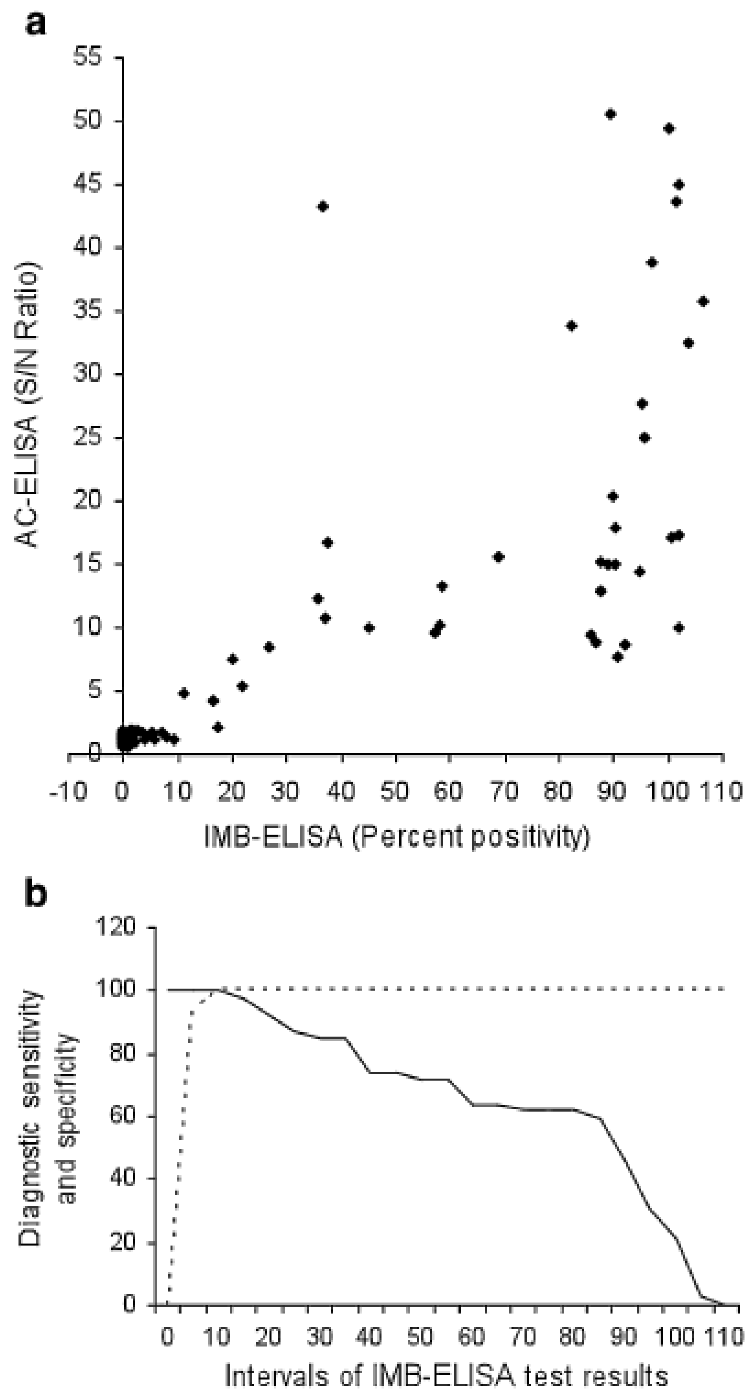

Fig. 2. Graphical representation of operator agreement when IMB-ELISA result was expressed as percent positivity. Boxed area highlights variation of diagnostic result between operators for samples close to the cut-off

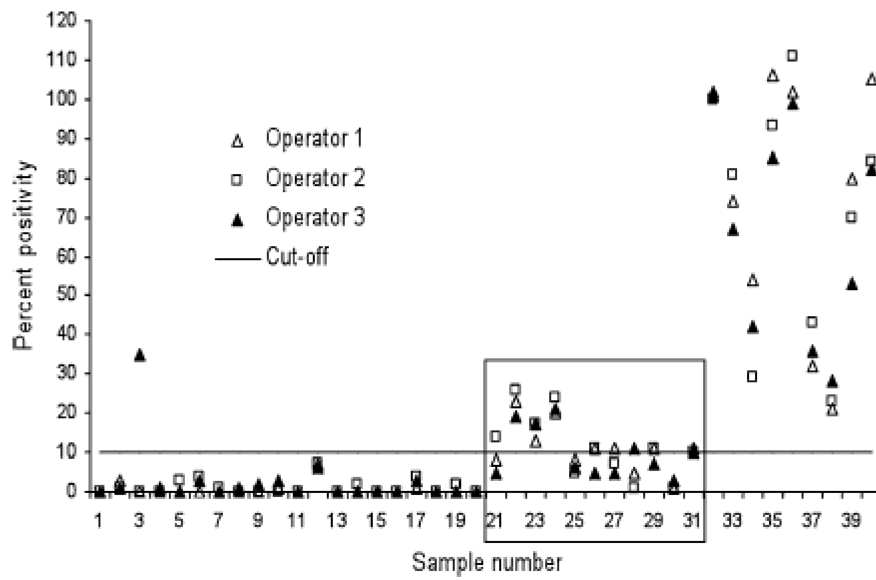

Table 1. Analytical sensitivity of the AC-ELISA and IMB-ELISA

Tissue sample	AC-ELISA	IMB-ELISA
Spleen	$7.08 \pm 1.1 \times 10^5$ (n = 2)	$2.21 \pm 0.37 \times 10^4$ (n = 2)
Tonsil	$1.58 \pm 0.0 \times 10^5$ (n = 1)	$2.22 \pm 0.35 \times 10^3$ (n = 2)
Kidney	Not detected	$2.81 \pm 0.0 \times 10^4$ (n = 1)

The detection limit of infectious virus in tissue expressed as $TCID_{50}$/100 μL

Table 2. Relative diagnostic sensitivity (DSn) and specificity (DSp), positive predictive value (PPV) and negative predictive value (NPV) of IMB-ELISA for the three operators to determine inter-operator variation using the AC-ELISA as the reference comparator

Operator	IMB-ELISA - result by eye (95% CI)				IMB-ELISA - Calculated as percent positivity (95% CI)			
	DSn	**DSp**	**PPV**	**NPV**	**DSn**	**DSp**	**PPV**	**NPV**
1	1.00 (0.73–1.00)	0.88 (0.69–0.97)	0.82 (0.56–0.95)	1.00 (0.82–1.00)	1.00 (0.73–1.00)	0.92 (0.73–0.99)	0.88 (0.60–0.98)	1.00 (0.83–1.00)
2	1.00 (0.73–1.00)	0.85 (0.75–0.97)	0.78 (0.52–0.93)	1.00 (0.82–1.00)	1.00 (0.73–1.00)	0.92 (0.73–0.99)	0.88 (0.60–0.98)	1.00 (0.83–1.00)
3	0.93 (0.64–1.00)	0.85 (0.64–0.95)	0.76 (0.50–0.92)	0.96 (0.76–1.00)	0.93 (0.64–1.00)	0.92 (0.73–0.99)	0.87 (0.58–0.98)	0.96 (0.78–1.00)

95% CI - 95% confidence interval

The samples tested (n = 40) comprised 20 negatives, 11 samples that approached the diagnostic cut-off and nine strong positives.