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Editors: J.V. Conlan, S.D. Blacksell, C.J. Morrissy and A. Colling



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Application of immunomagnetic bead technology for improved diagnosis of classical swine fever virus

James Conlan^{1,2,3}, Syseng Khounsy¹, Manivanh Phruaravanh¹, Vilaywan Soukvilai¹, Chris Morrissy^{1,2}, Axel Colling^{1,2}, Stuart Blacksell^{4,5}, Colin Wilks³ and Laurence Gleeson^{1,2,6}

Abstract

Classical swine fever (CSF) is a highly contagious viral disease of swine that causes major losses in all pig production systems in many regions of the world. In Lao PDR, CSF is endemic and outbreaks have adverse effects on the predominantly smallholder farming sector. Laboratory testing is required to accurately identify CSF outbreaks because of the difficulty of making a diagnosis based solely on clinical signs. The National Animal Health Centre, located in the national capital, Vientiane, has the capacity to reliably detect CSF antigen in tissue samples using an antigen capture (AC)-ELISA, and antibodies to CSF virus from serum samples using the complex trapping blocking ELISA. This paper describes the use of immunomagnetic beads (IMB) as the solid phase for the portable detection of CSF antigen in spleen samples and for the reliable detection of antibodies to CSF in animals vaccinated with a lapinised C-strain vaccine. The portable IMB-ELISA for antigen detection was shown to be 100% sensitive and 91% specific in comparison to the AC-ELISA. The IMB-Antibody-ELISA was shown to be 97% sensitive and 95% specific in comparison to the gold standard—neutralising peroxidase linked assay. These new diagnostic tests have the potential to improve CSF management through portable and rapid identification of outbreaks and the reliable and inexpensive monitoring of vaccination programs.

Introduction

Laboratory testing of clinical samples is of paramount importance if classical swine fever (CSF) outbreaks are to be correctly identified (Elbers et al. 2004; Paton and Greiser-Wilke 2003; van Oirschot

1999). Likewise, a rapid turnaround from sample collection to reporting results is necessary to ensure control measures are enacted in a timely manner. In Lao PDR sample submission can be delayed after collection, and delays also often occur once samples have been received at the laboratory. To counter this

¹ ACIAR Project AH/2003/001, National Animal Health Centre, Department of Livestock and Fisheries, Ministry of Agriculture and Forestry, Vientiane, Lao PDR

² CSIRO Livestock Industries, PO Bag 24, Geelong 3220, Victoria, Australia

³ School of Veterinary Science, The University of Melbourne, Parkville, Victoria, Australia

⁴ Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

⁵ Centre for Clinical Vaccinology and Tropical Medicine, Nuffield Department of Clinical Medicine, Churchill Hospital, University of Oxford, Oxford, United Kingdom

⁶ Now at FAO Regional Office for Asia and Pacific, Bangkok, Thailand

problem, new diagnostic tests have been developed to simplify the process, decrease reporting times and provide reproducible results in a portable format without compromising results (Conlan 2006; Conlan et al., in press). The use of immunomagnetic beads (IMB) for the detection of CSF virus has been described previously (Conlan 2006; Conlan et al., in press), where the results are read by eye without compromising test integrity.

Vaccination is a very important control measure for CSF; however, to maximise the potential benefit of a vaccination program, farmers and veterinary authorities need to be able to monitor herd immunity post vaccination. The neutralising peroxidase linked assay (NPLA) is the gold standard for the detection of antibodies to CSF virus (OIE 2004); however, this test is slow, laborious and expensive, and requires tissue culture facilities. In addition, the complex trapping blocking (CTB)-ELISA currently being used at the National Animal Health Centre (NAHC) is not able to be applied in monitoring vaccination programs. Therefore, rapid inexpensive alternatives to these two tests are required.

The results presented in this paper describe the application of the IMB-ELISA (Conlan 2006; Conlan et al., in press) in a near-to-field format for antigen detection, and its adaptation to a format for antibody detection, the IMB-Antibody (Ab)-ELISA.

IMB-ELISA for antigen detection

Control antigens

Negative and positive control antigen extracts were prepared as pooled 5% w/v homogenates in buffered detergent (1% Nonidet P-40 in phosphate buffered saline (PBS) with 5% normal goat serum (NGS) and 0.07% Proclin 300) (Conlan et al., in press).

IMB-ELISA method

Immunomagnetic beads (Spherotech Inc., USA) were coated with anti-pestivirus goat polyclonal antibody according to methods previously described (Conlan 2006; Conlan et al., in press), with some modification. The IMBs were coated at room temperature overnight instead of for 2 hours. Following coating, the IMBs were blocked, washed and resuspended to 0.20% w/v in storage buffer as previously described (Conlan 2006; Conlan et al., in press).

IMB-ELISA test kits were prepared in dropper bottles (Nalgene, USA) and reagents were prepared at working concentrations. Monoclonal antibody (MAb) 24/10 (Kosmidou et al. 1995), specific for CSF virus E2 protein, was incubated with an equal volume of filter-sterilised NGS for 20 minutes prior to dilution in buffer containing 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. Goat anti-mouse horseradish-peroxidase (HRP) conjugate (DakoCytomation, Denmark) was incubated with five volumes equivalent of NGS for 20 minutes prior to dilution in Guardian Peroxidase Stabiliser (Pierce, USA). The chromogen-substrate used in the test kit was TMB Liquid Substrate System (Sigma, USA) and was purchased as ready-to-use.

One drop (~35 μ L) of IMBs was added to 100 μ L of sample and controls in a 1.5 mL tube, mixed and incubated at room temperature (~25 °C) for 30 minutes. The tubes were placed on a magnet (Dexter Magnetic Technologies, USA) for 20 seconds and the supernatant discarded. Three drops (~100 μ L) of MAb were added, mixed and incubated at room temperature for 15 minutes. The tubes were again placed on the magnet, the MAb was discarded and three drops of conjugate were added, mixed and incubated at room temperature for 15 minutes. The tubes were placed on the magnet, the conjugate discarded and the IMBs were washed three times prior to transfer to a new tube. The final wash was removed and two drops (~65 μ L) of chromogen-substrate was added, mixed and incubated for 5 minutes at room temperature (Figure 1). Samples were considered positive if an obvious green/blue colour was visible (scored as 3+, 2+ or 1+ depending on intensity) and negative if the colour remained brown/red (scored as 0) (Figure 2).

Relative diagnostic performance

The relative diagnostic performance of the IMB-ELISA test kit was determined using an AC-ELISA (Fuqing et al. 2000; Shannon et al. 1993) as the reference comparator. During 2004–06, 110 spleen samples from CSF-suspected pigs were submitted to the NAHC, Vientiane. Specimens were transported in buffered glycerol (50% v/v glycerol in PBS), prepared as 5% w/v spleen homogenates in buffered detergent and stored at –85 °C prior to testing. The spleen samples were given a randomised number and tested in the IMB-ELISA test kit.

Kit stability

Four replicates of mid-positive and negative control antigens were tested in the IMB-ELISA at weeks 2, 4, 8 and 12 after preparing a kit. The kits were stored at 4 °C throughout. At the completion of the 5-minute chromogen-substrate incubation, 1N H₂SO₄ was added to stop the reaction, and the optical density was measured at a wavelength of 450 nm (OD₄₅₀).

Results

Of the 110 samples, 34 (31%) were positive by AC-ELISA and 41 (37%) were positive by IMB-ELISA. All samples positive by AC-ELISA were also positive by IMB-ELISA; that is, no false negatives were observed. The relative diagnostic per-

formance of the IMB-ELISA test kit was 100% (95%CI: 87–100) sensitive and 91% (95%CI: 81–96) specific in comparison to the AC-ELISA.

The kit was found to be stable for less than 3 months. After 12 weeks the detection efficiency as indicated by the optical density decreased substantially from an OD₄₅₀ of 0.80 to 0.36, a decrease of greater than 50%.

IMB-Antibody-ELISA for detection of antibodies to CSF virus

IMB-Antibody-ELISA method

The IMBs were coated, blocked and resuspended as described above to a final working concentration of 0.10% w/v. The MAb, conjugate and chromogen-

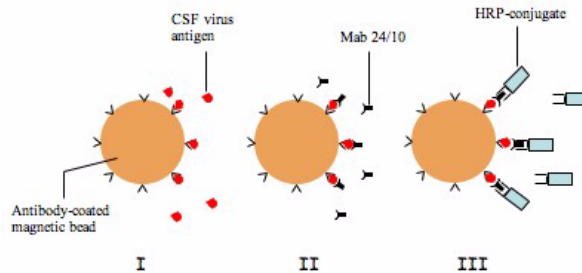


Figure 1. Principle of the IMB-ELISA for antigen detection. **I:** CSF virus antigen capture, **II:** specific detection by monoclonal antibody and **III:** chromogen-substrate colour development

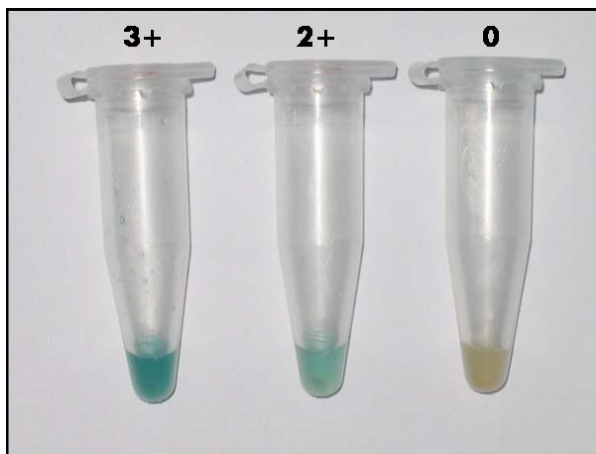


Figure 2. Colour range for test results; positive (bluest, score 3+) to negative (brownest, score 0)

substrate used were the same as those described above. The CSF virus antigen used in the blocking ELISA was the same as that described above diluted 1:2 to 2.5% w/v in buffered detergent.

Twenty-five μL of control or test serum was added to 25 μL of buffered detergent and incubated at room temperature for 5 minutes before the addition of 50 μL of CSF virus control antigen. The serum and antigen were mixed and incubated at 37 °C for 30 minutes followed by the addition of 50 μL of IMBs and shaking for 30 minutes at 37 °C. A no-serum control was included as OD_{max} . The tubes were placed on the magnet for 20 seconds, the supernatant was discarded and 100 μL of MAb added and shaken at 37 °C for 15 minutes. The MAb was removed and 100 μL of conjugate was added and shaken at 37 °C for 15 minutes. The IMBs were washed three times and transferred to a new tube, the final wash was removed and 50 μL of chromogen-substrate added and incubated at room temperature for 5 minutes. The reaction was stopped with 1N H_2SO_4 and the $\text{OD}_{450\text{nm}}$ measured; the percentage inhibition was calculated according to equation (1).

$$\text{PI} = 100 - \frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{max}}} \times 100 \quad \text{equation (1)}$$

Test samples

Twenty pigs were vaccinated with a lapinised C-strain CSF vaccine in two villages of Bolikhamxay province in central Lao PDR and bled at 0, 35 and 70 days post vaccination. In total, 57 serum samples were tested by NPLA (OIE 2004), Ceditest (CEDI Diagnostics, the Netherlands), CTB-ELISA (Blacksell 2001) and IMB-Antibody (Ab)-ELISA.

Data analysis

The diagnostic cut-off for the IMB-Ab-ELISA was visually assigned after graphically plotting the frequency against intervals of per cent inhibition for the 57 samples assessed, with the NPLA used as the reference test. The diagnostic performances of the IMB-Ab-ELISA and the CTB-ELISA were assessed by calculating relative diagnostic sensitivity and specificity using EpiCalc software (CDC, USA), with the NPLA and CEDI ELISA used as the reference comparators. The level of agreement of the tests was calculated using kappa statistic analysis (Smith 2006), where kappa scores of 0.41–0.60,

0.61–0.80, 0.81–0.99 and 1.00 correspond to levels of agreement of moderate, substantial, almost perfect and perfect, respectively.

Results

The diagnostic cut-off for the IMB-Ab-ELISA was set at greater than or equal to 50% inhibition (Figure 3). At this cut-off there were two false negatives and one false positive when compared to the NPLA. The relative diagnostic sensitivities and specificities of the IMB-Ab-ELISA and the CTB-ELISA are summarised in Table 1.

The levels of agreement between the CTB-ELISA and the CEDI ELISA, and between the CTB-ELISA and NPLA, were less than moderate. The IMB-Ab-ELISA showed almost perfect agreement with both the CEDI ELISA and NPLA.

General discussion

Antigen detection

Appropriate diagnostics are a critical component of CSF management and the speed and efficiency of application will, to a large degree, determine the outcome of a disease-control initiative. The research presented in this paper describes the application of IMB technology to CSF diagnosis in a portable and sensitive format suitable for use in the field. The detection of CSF viral antigen by IMB-ELISA was first described by Conlan et al. (in press), and was found to be a rapid, sensitive, specific and highly repeatable test format with demonstrated high levels of agreement between operators. Minimal training was required to implement the test in a laboratory and the test was not expensive. These combined factors make the test ideal for the conditions seen in a low-technology setting such as Lao PDR where sample submission from remote locations can be difficult. This research demonstrates that the test was successfully adapted to a portable format using dropper bottles for dispensing reagents, performing the test at room temperature and reading the result by eye. Diagnostic performance was good in comparison to an AC-ELISA, with 100% and 91% relative diagnostic sensitivity and specificity, respectively. The estimated shelf life was not as good as was expected using stabilised reagents. After 3 months the test performance dropped to unacceptable levels, and this will need to be corrected in the future.

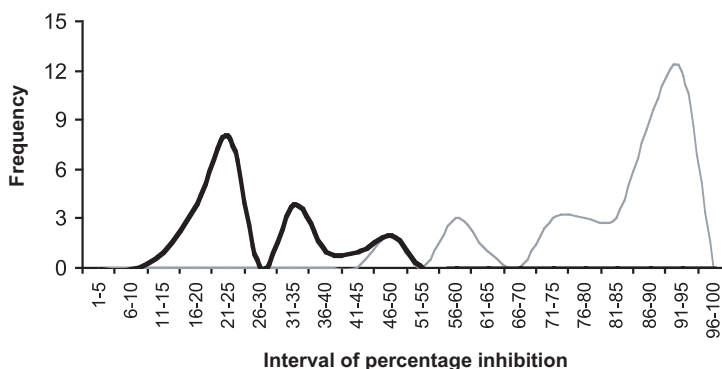


Figure 3. Analysis of the reactivity range of 57 serum samples tested in the IMB-Ab-ELISA. The black line represents samples negative for CSF antibodies (≤ 8 by NPLA) and the grey line represents samples positive for CSF antibodies (≥ 8 by NPLA).

Table 1. The relative diagnostic performance and level of agreement of the IMB-Antibody (Ab)-ELISA and complex trapping blocking (CTB)-ELISA in comparison with two standard antibody tests, the neutralising peroxidase linked assay (NPLA) and the Ceditest

	IMB-Ab-ELISA			CTB-ELISA		
	DSn	DSp	<i>K</i>	DSn	DSp	<i>K</i>
Ceditest	97	97	0.92	40	100	0.34
NPLA	97	95	0.92	39	100	0.32

DSn = diagnostic sensitivity; DSp = diagnostic specificity; *K* = kappa statistic

Monitoring vaccination

Vaccination is the only control and prevention measure undertaken in Lao PDR to minimise the occurrence of CSF in village production systems. A slaughter policy during an outbreak does not exist; however, in some villages, quarantine systems have been set up to decrease the risk of introducing disease into a village. Farmers and animal health officials are, therefore, highly reliant on the success of vaccine delivery and need suitable resources to accurately monitor vaccine uptake. The CTB-ELISA currently used in Lao PDR is not suitable for this purpose; this study demonstrated that its level of agreement with the NPLA test was very poor (0.32) and it showed low test sensitivity (39%) for the detection of vaccinated sero-positive animals. The adaptation of the IMB-ELISA into an antibody detection format has produced promising early results. The level of agreement with the NPLA was almost perfect (0.92) and the sensitivity and specificity were very high (97% and 95%, respectively). At this stage of test development, proof

of principle has been clearly demonstrated but too few samples have been tested to give a clear indication of test performance. Further work is required to adapt the test to a plate format to increase speed and the number of samples that can be tested.

Conclusions

Immunomagnetic bead technology is adaptable and versatile and can provide a platform for appropriate diagnostic test development in a limited-resource setting such as Lao PDR. The IMB-ELISA for CSF is inexpensive, portable, stable and a reliable test that requires minimal training to implement and will improve diagnostic services for pig farmers. The IMB-Ab-ELISA, while in the early stages of development, shows strong agreement with the 'gold standard' NPLA and could be a valuable tool for monitoring vaccine uptake.

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