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Development and validation of a 3ABC antibody ELISA in Australia for foot and mouth disease

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Objective To measure the diagnostic performance of an Australian-developed ELISA for the detection of antibodies against the non-structural proteins (NSP) 3ABC of the foot and mouth disease (FMD) virus.

Design Test development and validation study.

Methods The diagnostic specificity was determined using 2535 sera from naïve animals and 1112 sera from vaccinated animals. Diagnostic sensitivity was calculated from the data for 995 sera from experimentally and field-infected animals from FMD-endemic countries in South East Asia. A commercial ELISA detecting antibodies against FMD virus NSP was used as the reference test to establish relative sensitivity and specificity. Bayesian latent class analysis was performed to corroborate results. The diagnostic window and rate of detection were determined at different times using sera from cattle, sheep and pigs before and after infection, and after vaccination and subsequent infection. Repeatability and reproducibility data were established.

Results At 35% test cut-off, the 3ABC ELISA had an overall diagnostic sensitivity of 91.5% and diagnostic specificity of 96.4%. The diagnostic sensitivity in vaccinated and subsequently infected cattle was 68.4% and diagnostic specificity in vaccinated cattle was 98.0%.

Conclusions The 3ABC ELISA identified field and experimentally infected animals, as well as vaccinated and subsequently infected animals. Diagnostic sensitivity and specificity estimates for other FMD NSP tests are comparable with the results obtained in this study. This NSP ELISA was found to be 'fit for purpose' as a screening assay at the herd level to detect viral infection and also to substantiate absence of infection.

Keywords 3ABC ELISA; foot and mouth disease; screening tests

Abbreviations ASe, analytical sensitivity; ASp, analytical specificity; CI, confidence interval; CV, coefficient of variation; dpi, days post infection; DSe, diagnostic sensitivity; DSp, diagnostic specificity; FMD, foot and mouth disease; NSP, non-structural protein; PI, percentage of inhibition; ROC, receiver-operating characteristic; SD, standard deviation; SPCE, solid-phase competition ELISA

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Foot and mouth disease (FMD) must be differentiated from other vesicular diseases, so rapid laboratory confirmation of a suspected case is imperative. Direct diagnosis is by virus isolation or demonstration of antigen or nucleic acid in samples of tissue or fluid. Detection of antibodies to FMD viral non-structural proteins (NSPs) is an indicator of infection, irrespective of vaccination status,[1] and is particularly useful in countries where vaccination is used or in mild cases or where epithelial tissue cannot be collected. Most of the commercial tests for the detection of antibodies to NSPs use recombinant expressed NSP 3ABC target antigens.[2] Antibodies against NSP indicate past or present infection with any of the seven serotypes of FMD virus, even if animals have been vaccinated. Recent publications indicate that the sensitivity and specificity of such assays for the detection of carriers among vaccinated cattle populations are approximately 90% and 99%, respectively.[1,3-8] The use of NSP-ELISAs to substantiate freedom from FMD infection after emergency vaccination of cattle has become an important option in Europe in recent years.[9]

Vaccine purity is an important consideration when using these assays because trace amounts of NSP in formulations may result in false-positive reactions in animals that are repeatedly vaccinated.[1,10] Conversely, antibodies against NSPs are not detected by some assays[2,11] in some FMD-vaccinated animals that have been subsequently challenged with infectious virus and then confirmed to be persistently infected. Paradoxically, in some vaccinated cattle that later become infected, there is a delayed antibody response against NSP compared with the response in unvaccinated-and-infected cattle.[2,5] Some studies have shown that anti-NSP tests can identify carriers in the absence of any previous clinical signs when virus is not detectable by reverse transcriptase polymerase chain reaction, suggesting that anti-NSP testing, even of individual animals, may detect infections that may be missed by other tests.[2,12] For these reasons, NSP assays are best used at the herd level to detect FMD virus circulation in vaccinated populations.

For FMD-free countries such as Australia, it is important to develop and validate test platforms based on a differentiating-infected-from-vaccinated-animals (DIVA) strategy and to be prepared for FMD incursions by having an independent supply of reagents. The aim of this study was to assess an Australian-developed 3ABC ELISA for

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its 'fitness for purpose' (as defined in the OIE *Manual of diagnostic tests and vaccines for terrestrial animals: validation and certification of diagnostic kits*)[13] to confirm suspected cases of FMD and to substantiate absence of infection during serosurveillance and proof-of-freedom testing after an outbreak.

Materials and methods

3ABC reagent development

A novel aspect in the development of this ELISA was the production of IgY antibodies against NSP by vaccination of chickens with *E. coli*-expressed 3ABC. Briefly, a recombinant baculovirus containing the FMD virus (strain C-Turup) 3ABC genes[14] was supplied by the Danish Veterinary Institute for Virus Research to produce the ELISA antigen. Crude soluble protein was titrated onto a microplate (Nunc Maxisorp, Thermo Fisher Scientific, Waltham, MA, USA) to determine the optimal concentration for binding antibodies against NSP, while minimising non-specific cross-reactivity. Briefly, 3ABC cDNA (FMD virus serotype O 1 K, provided by the World Reference Laboratory, Pirbright, UK) was cloned into the pRSETb expression vector according to the manufacturer's protocol (Invitrogen, San Diego, CA, USA). The expressed 3ABC was purified using sodium dodecyl sulfate polyacrylamide gel electrophoresis. Specific pathogen-free chickens were vaccinated with 100 µg of purified antigen and eggs were collected from 2 weeks post inoculation for a period of 3 months. The IgY antibodies were isolated from the egg yolks by chloroform extraction according to Polson[15] and pooled to minimise any batch-to-batch variation.

3ABC ELISA

A competitive ELISA was regarded as the most suitable format because it can be used to test all sera from all susceptible species. Positive cattle sera controls were obtained from the World Reference Laboratory, UK, and Lelystad, the Netherlands, from animals infected experimentally by intradermal inoculation of the tongue three times with FMD virus strains A 22 Iraq, Asia 1 Shamir and C 1 Detmold. A positive serum control was obtained from a sheep infected experimentally by intradermal inoculation with strains O 1 Tunisia, A 5 Bernbeuren and A 5 Westerwald. A positive pig serum sample was obtained from a minipig experimentally infected by intradermal inoculation of the bulbus of the heel and intravenous inoculation with the O 1 Taiwan strain. Negative control sera were pooled from samples from Australian cattle, sheep and goats.

The ELISA was developed under standard laboratory conditions with all incubation steps at 37°C with gentle orbital shaking unless otherwise stated. Plates were washed five times between incubation steps with Dulbecco's phosphate-buffered saline. Briefly, 96-well Nunc Immuno Maxisorp plates were coated with 50 µL/well of baculovirus-expressed 3ABC diluted in 0.05 mol/L carbonate/bicarbonate buffer (pH 9.6) for 1 h. After washing, the wells were blocked for 1 h by the addition of 100 µL of blocking buffer (phosphate-buffered saline containing 0.05% Tween 20, 10% horse serum and 5% skimmed milk powder). After washing, 50 µL of the test or control sera were added at a 1 : 5 dilution (in blocking buffer) and the plate was incubated for 30 min. Purified IgY (50 µL/well) was then added directly to the test/control sera and the plate incubated for an additional 30 min. After washing, anti-chicken IgY horseradish peroxidase conjugate was added (50 µL/well in blocking buffer) and incubated for 1 h. After washing, 50 µL/well of TMB substrate (T0440, Sigma, St Louis, MO, USA) were added and the reaction stopped after 10 min at room temperature by the addition of 50 µL/well of 1 mol/L H_2SO_4 and the absorbance of each well at 450 nm measured on an ELISA reader after blanking. Sera were tested singly or duplicate. When tested in duplicate, the final result was expressed as the mean value. Each plate contained four replicates of the positive and negative controls and two replicates of conjugate only controls and controls that contained no inhibitory serum (OD max). In general, results were obtained from the first testing and not from retested sera (i.e. when internal controls were outside acceptance limits). Results were expressed as percentage of inhibition (PI) using the mean value (or single test value) from the test serum replicates in the formula: $100 - (100 \times (\text{test serum})/\text{mean OD max})$.

SPCE and LPBE

The solid-phase competition ELISA (SPCE) and liquid-phase blocking ELISA were used as described in the 2008 OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*[1] and Paiba et al,[16] respectively.

PRIOCHECK® FMDV NS ELISA

The PRIOCHECK® FMDV NS ELISA was used according to the manufacturer's instructions (Prionics AG, Schlieren-Zuerich, Switzerland).[2,5]

Samples

A total of 4984 sera from 11 countries were tested. Field sera were collected from FMD-free areas (Australia) and from FMD-endemic regions as post-outbreak samples from the Lao PDR, Malaysia, the Philippines, Thailand and Vietnam (animals with clinical signs or in-contact animals). Sera were also available from serial blood samples from experimentally infected or vaccinated animals and serial or single blood samples from FMD reference laboratories in Argentina, Hong Kong, Israel, the UK and the USA. Some of these serum samples were from the same animal and provided data on the diagnostic window and rate of detection of antibody, but multiple samples from the same animal were not used to estimate diagnostic sensitivity (DSe) or specificity (DSp) as they could not be regarded as independent observations. Nevertheless, when individual blood samples were available for several months after infection, samples were chosen from within the typical post-outbreak 'proof-of-freedom' testing interval (21 days to ≥3 months).

Cut-off selection and estimation of sensitivity and specificity

The cut-off for cattle was selected using sera from naïve, vaccinated and infected animals using receiver-operating characteristic (ROC) analysis.[17] Results from the naïve populations were used to calculate DSp-based cut-offs at the mean plus three standard deviations (SD) and the 99th percentile.[18]

Analytical sensitivity and specificity of 3ABC ELISA

The analytical sensitivity (ASe) of the 3ABC ELISA was assessed by determining the endpoint dilution of a positive bovine control serum using a two-fold dilution series from 1/5 to 1/640. The endpoint was

the dilution at which the value for the test sample was the same as that of the negative control. The earliest times for the detection of serological responses after infection and the duration of the antibody response were assessed using serial serum samples collected from experimental infections of 21 cattle in Argentina, 3 sheep at the WRL and 4 pigs infected with an Asia 1 strain from the Plum Island Laboratory, USA.

The analytical specificity (Asp) was assessed using sera from cattle that had been infected with malignant catarrhal fever virus, the New Jersey, Indiana and Piry Bean strains of vesicular stomatitis virus, bovine viral diarrhoea virus and sera from pigs infected with swine vesicular disease virus.

Experimentally vaccinated Australian cattle, sheep and pigs and Argentinian cattle were serially tested at different time intervals to determine the specificity of the 3ABC ELISA in vaccinated animals. The SPCE was used to measure the antibody response to FMD viral structural proteins.

Diagnostic sensitivity

The DSe was calculated using sera from naturally and experimentally infected animals. The post-outbreak cattle from Lao PDR, Thailand and Vietnam were confirmed as having been infected using the PRIOCHECK® FMDV NS test. Experimentally infected animals were defined based on clinical signs and diagnostic results from the laboratories supplying the sera. Experimentally derived sera were from infected sheep, and vaccinated and subsequently infected cattle, sheep and pigs from the WRL; sera from infected cattle from the Animal Health Service and National Institute of Agriculture in Argentina; and sera from infected pigs from the US Department of Agriculture laboratory at Plum Island. No repeat blood samples were included from the same individual to calculate the DSe. When serial blood samples were available, only one sample was selected. Sera collected 3–4 weeks after infection or later were used in estimating the DSe for proof-of-freedom testing after an outbreak.

Diagnostic specificity

Sera from naïve animals were available from Australia and from day 0 blood samples from experimentally vaccinated-and-infected animals. In addition, sera from South East Asian countries in which FMD is endemic that tested negative in the PRIOCHECK® FMDV NS test were used to estimate the relative DSp of the 3ABC ELISA. Sera from vaccinated animals were selected at times when seroconversion would be expected (≥21 days after vaccination) and seroconversion was confirmed using SPCE.

Precision

Repeatability estimates, including upper and lower limits for positive and negative serum controls, were established after 48 repetitions of the ELISA. Estimates for measurement of uncertainty were calculated using a 'top-down' or 'control-sample' approach.[19] A proficiency test round, which included six samples, was performed by seven Australian state laboratories to obtain estimates for reproducibility and repeatability.

Data analysis

Standard statistical analyses were performed using Microsoft Office Excel 2003 and MedCalc Version 9.6.4.0. Data for the samples from cattle from South East Asia were also analysed using a Bayesian approach based on latent class models.[20-22]

For these analyses, counts of ++, +−, −+ and −− were recorded separately for Vietnam, Lao PDR and Thailand, where ++ indicates a positive result in both tests; −+ a negative result in the 3ABC and a positive result in the PRIOCHECK® FMDV NS test; +− a positive result in the 3ABC and a negative result in the PRIOCHECK® FMDV NS test; and −− is negative result in both tests. The full set of data then constituted three 2 × 2 contingency tables, where the 2 × 2 tables of counts for each country were assumed to have independent multinomial distributions. No assumption was made that either test was a reference standard and both tests were assumed to have imperfect sensitivity and specificity. Prevalences were assumed to be distinct from country to country, with the required assumption that at least two of the three prevalences were distinct. The assumption was that the tests would be dependent (conditional on disease status) because they had the same biological basis, that is, the detection of antibodies against FMD viral NSP. Using the Bayesian approach, information about the PRIOCHECK® FMDV NS ELISA's sensitivity and specificity was modelled using independent and informative beta distributions.[2,20] Prior information for the prevalences, for test sensitivity and specificity of the 3ABC ELISA and for the dependence parameters was all specified as independent uniform distributions. Bayesian inferences were based on the joint posterior distribution, which was numerically approximated using WinBUGS (http://www.mrc-bsu.cam.ac.uk/bugs/). Final inferences were given as the median of the 2.5% and 97.5% quantiles of the marginal posterior distributions for each of the parameters; these constituted a point estimate and a 95% probability interval, respectively. Sensitivity analyses were performed to check the effect of the priors on the final results.

Results

Analytical sensitivity

The positive control serum yielded high PI values (≈ 80%) down to a 1/20 dilution after which the PI values gradually decreased. Inhibition was still seen at dilutions between 1/160 and 1/320, whereas at 1/640 the level of inhibition was indistinguishable from that seen in negative control samples.

Analytical specificity

There was no detectable inhibition by sera from animals infected with other diagnostically relevant pathogens at a 1/5 dilution. Thus, the ASp of the 3ABC ELISA was 100% in this study.

Cut-off

Preliminary cut-off points were selected per species with reference to the frequency distribution by calculating the mean + 3 SD and the 99th percentile for samples from Australian animals, as indicated in Table 1 and by ROC analysis for cattle (i.e. 35%) (Figures 1, 2).

Naïve, vaccinated, infected and vaccinated-and-infected animals

Robust estimates for the DSp and DSe for various categories of cattle samples are shown in Table 2. For goats, sheep and pigs, results are also presented, but these were based on substantially fewer animals.

Table 1. Mean percentage of inhibition (PI) values of a 3ABC ELISA for naïve Australian livestock species plus 2 and 3 standard deviations (SD) and 99th percentile for cut-off estimates

Species	No. tested	Mean PI	Mean PI + 2SD	Mean PI + 3SD	99th percentile
Cattle	1340	8	26	35	33
Goats	112	8	36	50	40
Sheep	461	8	32	44	46
Pigs	367	6	32	45	32
Deer	16	1	9	13	5

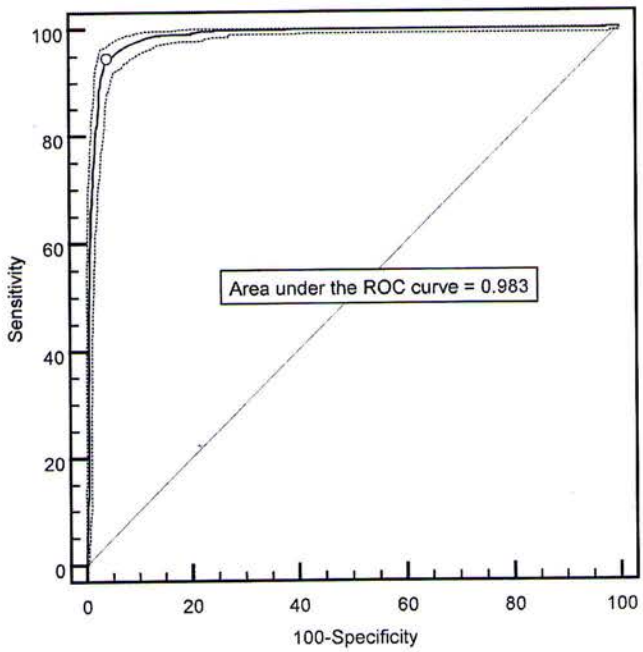

Figure 1. ROC analysis for a 3ABC ELISA with results from a total of 2572 cattle sera. Dotted lines represent 95% confidence interval and the circle in the upper left corner is the highest Youden index indicating the best combined sensitivity and specificity. ROC, receiver-operating characteristic.

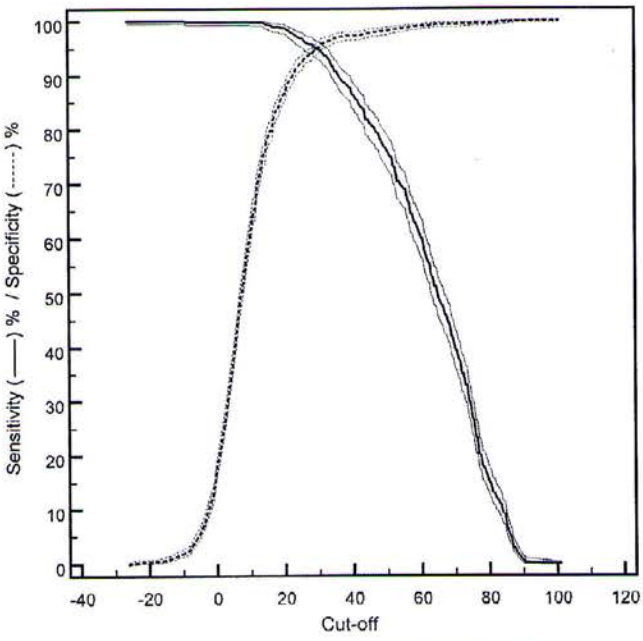

Figure 2. Plot-versus-criterion-analysis for a 3ABC ELISA. The same data set as in Figure 1 were used to plot sensitivity (continued line) and specificity (dotted line) with their respective 95% confidence intervals (y-axis) at different cut-offs (x-axis). A higher cut-off increases specificity and decreases sensitivity; a lower cut-off decreases specificity and increases sensitivity.

Naïve animals. The overall DSp in naïve cattle was 96.4% (95% confidence interval (CI) 95.3–97.2%), so 58 of 1595 negative cattle were misclassified as positive by the 3ABC ELISA (Table 2). These samples were from uninfected-unvaccinated animals. Of the 1356 naïve Australian animals, the majority were cattle (1340) and of them 1331 were negative in the 3ABC ELISA at a 35% cut-off (Table 1). In contrast, the relative Sp of the 3ABC ELISA for cattle sera from the Lao PDR, Thailand and Vietnam tested by the PRIOCHECK® FMDV NS ELISA was 73%, 54% and 94%, respectively (Table 4). Specificity estimates for sera from naïve goats, sheep and pigs were between 98 and 99% (Tables 1, 2).

Vaccinated animals. The overall DSp for vaccinated cattle was 98.0% (95% CI 95.0–99.5%) with the 3ABC ELISA (Table 2). The DSp for vaccinated cattle was slightly higher than for naïve cattle (98% vs 96%). In contrast to the naïve cattle, no major difference in DSp was seen between different countries or groups for vaccinated animals. Animals in all groups developed an antibody response to structural FMD viral antigens in the SPCE as soon as 7 days after vaccination (data not shown).

The DSp estimates for vaccinated sheep and pigs were approximately 100% and the specificity for vaccinated goats from Malaysia was estimated to be 5% (Table 2).

Infected animals. The overall sensitivity of the 3ABC ELISA in cattle was 91.5% (95% CI 89.3–93.4%) (Table 2). Results from repeatedly tested cattle were used to determine the rate of detection using serial samples collected 7–535 dpi. At a preliminary cut-off of 35%, 4 of 10 cattle (40%) were positive in the very early stage (7–10 dpi), 5 of 6 cattle (83%) in the early stage (11–30 dpi) and all 7 cattle tested positive in the late stage (31–100 dpi), which corresponds to the

Table 2. Numbers of naïve, vaccinated, infected and vaccinated-infected animals and estimates for sensitivity and specificity for a 3ABC ELISA (35% cut-off)

Species	Category	No. of positive (Se) or negative (Sp)	No. tested	Sensitivity (Se) Specificity (Sp)
Cattle	Naïve	1537	1595	96.4 (Sp)
	Vaccinated	198	202	98.0 (Sp)
	Infected	709	775	91.5 (Se)
	Vaccinated-infected	39	57	68.4 (Se)
Goats	Naïve	110	112	98.2 (Sp)
	Vaccinated	1	20	5.0 (Sp)
	Infected	NA	NA	NA
	Vaccinated-infected	4	7	57.1 (Se)
Sheep	Naïve	454	461	98.5 (Sp)
	Vaccinated	26	26	100.0 (Sp)
	Infected	3	3	100.0 (Se)
	Vaccinated-infected	6	7	85.7 (Se)
Pigs	Naïve	364	367	99.2 (Sp)
	Vaccinated	277	278	99.6 (Sp)
	Infected	12	23	52.2 (Se)
	Vaccinated-infected	0	8	0.00 (Se)

NA, not available.

approximate period for serosurveillance after emergency vaccination (Table 3).

Sera from the Lao PDR, Thailand and Vietnam were collected after outbreaks caused by serotypes A, Asia 1 and O, and samples were tested with the PRIOCHECK® FMDV NS test as a reference method. The 3ABC ELISA had relative diagnostic sensitivities of 92%, 94% and 95%, respectively, for these serotypes (Table 4).

Results from the Bayesian latent class analysis are given in Table 5. The estimated sensitivity for the 3ABC ELISA was 92% (95% CI 88–95%), and the specificity estimate was 97% (CI 95% 91–99%). Prevalences in the Lao PDR and Thailand appeared similar, but distinct from the prevalence in Vietnam.

It has been reported that the best estimate independent of the current data of the sensitivity of the PRIOCHECK® FMDV NS ELISA is 97%, with 95% certainty that it was at least 90%. The best estimate for its specificity is 98%, with 95% certainty that it was at least 95%. These specifications were translated to beta (53.6, 2.6) and beta (151.8, 4.1) distributions.[21] The only other prior knowledge incorporated into the analysis was that the prevalences were all at least 25% and that the 3ABC test accuracies were both at least 50%. Aside from that, all other values were assumed to be 'a priori' equally likely, so these priors should have little effect on the final analysis. Two additional parameters, the sensitivity and specificity covariances, were also assessed.[22]

From these studies we were able to define corresponding correlations between the two tests, where values of zero correspond to no (conditional) dependence and values near −1 or 1 correspond to near perfect negative and positive (conditional) dependence, respectively. The prior information on the sensitivity and specificity covariance was uniform and thus not informative. The posterior estimates of the correlations between the two tests, conditional on animals being diseased or healthy, were 0.16 (−0.04, 0.50) and 0.42 (0.012, 0.88), respectively. Thus, there was clear evidence of dependence among the

Table 3. Rate of detection of FMD NSP by 3ABC ELISA in sera from vaccinated-infected cattle and sheep and unvaccinated experimentally infected cattle at different time intervals after infection (very early stage 0–10 dpi; early stage 11–30 dpi; late stage 31–100 dpi)

Stage of infection	Sensitivity (%) (95% CI)
Vaccinated-infected cattle	
O Manisa very early stage (17)*	18 (4, 43)
Asia 1 early stage (14)	93 (66, 100)
A 24 early stage (12)	67 (35, 90)
Asia 1 late stage (7)	86 (42, 100)
O Manisa late stage (32)	53 (35, 71)
SAT 2 late stage (9)	89 (52, 100)
A Iran late stage (7)	100 (59, 100)
Vaccinated-infected sheep	
O Manisa, O UKG2001 late stage (7)	86 (42, 100)
Experimentally infected cattle (no prior vaccination)	
Early phase (10)	40 (12, 74)
Mid phase (6)	83 (36, 100)
Late phase (7)	100 (59, 100)

*Numbers in parentheses in left column represents number of available animals for this category. dpi, days post infection; FMD, foot and mouth disease; NSP, non-structural proteins.

healthy cattle and a reasonable inference that this was also the case among the diseased cattle. A sensitivity analysis was performed in which the prior probability distributions for the sensitivity and specificity of the PRIOCHECK® FMDV NS ELISA were down-weighted by a factor of 2 and this was found to result in little difference in the inferences. A second perturbation was made in which the best prior estimates of sensitivity and specificity were reduced to 95% and 96%, respectively, and where there was 95% certainty that the true values were at least 80% and 90%, respectively. In this case, the posterior estimates of sensitivity and specificity dropped by approximately 1% and the corresponding estimates for the PRIOCHECK® FMDV NS ELISA dropped by 1% and 2%, respectively. The data were also analysed under a model of conditional independence and the point estimates for sensitivities were 1% and 2% higher, but other estimates were otherwise very similar to those obtained in the dependence model. The main assumptions for the validity of this analysis were that the test accuracies would be similar across countries and that the prior information about the PRIOCHECK® FMDV NS ELISA test was reasonably accurate (Table 5). Sensitivity estimates for infected sheep and pigs were 100% (3/3) and 52% (12/23), respectively (Table 2). No data were available for infected goats.

Table 4. Relative diagnostic sensitivity and specificity of a 3ABC ELISA compared with PRIOCHECK® NS ELISA in cattle populations in the Lao PDR (91.8%, 72.7%), Thailand (94.0%, 54.3%) and Vietnam (95.1%, 94.4%), respectively (PRIOCHECK® FMDV NS ELISA 50% cut-off; 3ABC ELISA 35% cut-off)

		PRIOCHECK® FMDV NS ELISA		
		(+)	(–)	TOTAL
The Lao PDR				
3ABC ELISA	(+)	247	9	
	(–)	22	24	
Total		269	33	302
Thailand				
3ABC ELISA	(+)	172	16	
	(–)	11	19	
Total		183	35	218
Vietnam				
3ABC ELISA	(+)	194	8	
	(–)	10	136	
Total		204	144	348

Vaccinated-and-infected animals. Results for vaccinated-and-infected cattle, goats, sheep and pigs are summarised in Table 2. For calculation of the DSe in cattle, sera from 57 cattle were available for the relevant surveillance period of 28–90 dpi: 39 were positive in the 3ABC ELISA, giving a DSe of 68%. In goats, 4 of 7 vaccinated-and-infected animals from Malaysia were positive in the 3ABC ELISA, giving a DSe of 57%. The two groups of pigs vaccinated and then infected with O Manisa and O UKG were negative in the 3ABC ELISA, giving a DSe of 0%.

A further breakdown for cattle and sheep into three different time intervals is presented in Table 3. The lowest detection rate of 18% was obtained in the very early stage for O Manisa with vaccinated and then infected cattle. Early stage detection rates were 93% or 67%, respectively, for cattle infected with Asia 1 or A 24. In the four groups of cattle vaccinated and infected with Asia 1, O Manisa, SAT 2 and A Iran, the rates of detection for the late stage were 86%, 53%, 89% and 100%, respectively.

Sera from a group of vaccinated (O Manisa) and subsequently infected (O UKG 2001) sheep were available only for the late serosurveillance period of 31–100 days (40 dpi); 6 of the 7 were positive in the 3ABC ELISA, a detection rate of 86%, which was comparable to the corresponding results for cattle (Tables 2, 3).

ROC analysis

ROC curve analysis was performed using sera from naïve, vaccinated and infected cattle. For this analysis, naïve and vaccinated cattle were given a negative status (0) and infected cattle had status of 1. The area under the ROC curve was 0.98 (Figure 1), which is considered 'highly accurate'.[17]

A modified ROC analysis was made to illustrate all possible combinations of sensitivity and specificity at all possible cut-off values (Figure 2). At a PI of 35%, the DSe and DSp were 91.6% and 96.6%, respectively.

Precision

Upper and lower control limits for the 3ABC ELISA were established for the OD max for the antigen, the strong positive (C++) serum and the negative (C–) control serum. The average and the coefficient of variation (CV) of the 3ABC ELISA were assessed after 47 independent replications between July 2006 and March 2009. Results for OD max, C++ and C– were 1.29 (CV 38.04), 85.85% (CV 6.7%) and 4.7% (CV 225.9%), respectively. The uncertainty of the measurement was estimated at the cut-off point using a top-down or control-sample approach and resulted in a range of 30–40 PI.[19] In 2013, a proficiency

Table 5. Sensitivity (Se) and specificity (Sp) of 3ABC and PRIOCHECK® FMDV NS ELISAs and prevalence estimates for cattle sera from the Lao PDR, Vietnam and Thailand using Bayesian latent class analysis

Quantile (%)	Se 3ABC	Sp 3ABC	Se PRIOCHECK	Sp PRIOCHECK	Prev Lao	Prev Vietnam	Prev Thailand
2.5	87.9	91.0	89.8	94.6	88.9	54.8	86.8
50	92.3	97.1	94.2	97.7	93.1	60.6	92.1
97.5	94.9	99.8	96.6	99.3	98.2	66.5	97.5

test round, which included 6 samples, was performed in 7 Australian state laboratories. Results indicated acceptable reproducibility and repeatability estimates for the 3ABC ELISA.

Discussion

The 3ABC ELISA achieved good discrimination between infected animals and naïve/vaccinated and vaccinated-and-infected animals. Because of the limited numbers of samples available for assessment, only preliminary Se and Sp estimates could be determined for goats, sheep and pigs. Robust estimates were obtained for cattle and were in agreement with those obtained with commercial NSP-ELISAs at 21–180 dpi (DSe 88.9–93.7% and DSp 97.3–98.1%).[2–8]

The 3ABC ELISA was assessed as a screening test with a lower cut-off (35%), which increased sensitivity and decreased specificity. This cut-off affected the relative specificity compared with the PRIOCHECK® FMDV NS test. Although both tests use baculovirus-expressed 3ABC antigen in a competitive format, the 3ABC ELISA used a chicken anti-3ABC polyclonal detector serum (followed by a polyclonal rabbit anti-chicken horseradish peroxidase conjugate) whereas the PRIOCHECK® FMDV NS test used a labeled anti-FMD viral NSP 3B monoclonal antibody as detector. The polyclonal detector antibody in the 3ABC ELISA was used to maximise reaction with all three NSPs and is likely to increase the number of false-positive results and decrease the relative DSp compared with the PRIOCHECK® FMDV NS test, which mainly detects antibodies against FMD viral NSP 3B.

Previous studies have suggested that natural infections tend to elicit a more heterologous immunological response than experimental ones and that estimates of sensitivity based on experimentally infected animals are likely to be overestimates of the sensitivity in naturally infected animals.[18] For that reason samples from field-infected animals in the Lao PDR, Thailand and Vietnam were used to estimate sensitivity. For example, FMD viral serotypes O, A and Asia 1 were confirmed as the causes of FMD outbreaks in the Lao PDR between 1996 and 2006.[23] In the Lao PDR, vaccination is not used routinely to prevent FMD, but as an emergency response to control spread. In Vietnam and Thailand vaccination is used routinely and the chance of vaccinated cattle becoming infected during an outbreak is higher. One reason for the better overall agreement between the two NSP-ELISAs for the post-outbreak sera from Vietnam could be that these samples were tested at the same time and location. In contrast, testing of the sera from Thailand and the Lao PDR was performed several years apart and at different locations.

DSp in vaccinated animals

Cattle, sheep and pigs vaccinated at the Australian Animal Health Laboratory, Geelong, Victoria, Australia, developed measurable antibody responses to structural FMD viral proteins in the SPCE but none showed a significant antibody response in the 3ABC ELISA (data not shown). Weak positive results observed in some cattle vaccinated with A 22 Iraq and O1 Manisa were not confirmed on resampling, an approach recommended to eliminate weak false-positive reactors.[23] The specificity estimates of the 3ABC ELISA in vaccinated animals were consistent with results from other studies, which suggests that higher DSp in vaccinated than in naïve cattle can be attributed to a more accurate assessment of the status of animals before vaccination.[2,3]

In our study, many of the serum samples from supposedly naïve or uninfected cattle were obtained from field surveys with a limited history about previous FMD infection or vaccination. In contrast, high specificity estimates for Australian livestock matched results from a study in New Zealand where testing of two commercial 3ABC ELISAs suggested specificities higher than 99% for cattle, sheep and pigs.[23]

DSe in infected animals

The estimates of DSe and DSp for cattle based on the Bayesian latent class model on data for all three South East Asian countries were 92% and 97%, respectively. This analysis had the advantage of not assuming that the PRIOCHECK® FMDV NS test was a perfect reference test and provided probability intervals that indicated the uncertainty in the estimates (Table 5).

Numbers of samples from other infected species were too small for statistical analysis but were used to provide an initial estimate of sensitivity (Table 2).

Sensitivity in vaccinated-and-infected animals

As a general observation, it can be concluded that detection rates in vaccinated-and-infected animals were considerably lower than in unvaccinated-and-infected animals (Tables 2, 3).

A decrease in the sensitivity in vaccinated-and-infected animals has been observed for NSP-ELISAs.[2] Although there was some variation in the magnitude of results in the present study, the 3ABC ELISA gave positive results for all serotypes used in this evaluation (Table 3).

Experimentally infected animals

The purpose of testing experimentally infected animals was to obtain answers to three important questions: how early, for how long and at what detection rate was the 3ABC ELISA able to identify infected animals. The 3ABC ELISA was able to detect antibodies with increasing confidence in the early, mid and late stages of infection in different target species and for an extended period of time.

Conclusions

The Australian 3ABC ELISA was found to be a suitable screening assay at the herd level to detect evidence of FMD infection and also to substantiate absence of infection. Although the DSe and DSp of NSP-ELISAs are important when substantiating freedom from FMD, other factors such as prevalence of infection, herd and sample size, population characteristics and survey design are equally important.

Because of Australia's freedom from FMD, livestock would probably receive only one or two vaccinations with highly purified FMD virus in the event of an outbreak. Results from this study indicate that the proportion of animals with detectable concentrations of vaccine-induced anti-NSP antibodies would be very low.[2,9,10] However, after an outbreak, during proof-of-freedom testing, the prevalence of infected animals can decrease quickly to close to zero. Low prevalences have a major effect on the positive predictive value of a diagnostic test, even

if it has a high specificity. Appropriate testing algorithms, including retesting, resampling, and serial or parallel serology can help decrease the rate of false-positive or false-negative results; that is, the use of two ELISAs in series has been suggested as an approach to improve the overall specificity during serosurveillance to re-establish freedom of FMD.[24]

During FMD simulation exercises in Australia, the 3ABC ELISA has been tested on a robotic platform and its capacity was estimated to be approximately 10,000 samples per day. One advantage of the 3ABC ELISA over the PRIOCHECK® FMDV NS ELISA is a reduced turn-around time, with results available within less than 4 h compared with an overnight incubation step in the latter.

Test reagents and a standard operating procedure are available and have been used effectively in overseas laboratories during FMD outbreaks in the Lao PDR, Thailand and Vietnam. In Australia, the 3ABC ELISA has been used during exotic disease exclusion testing and preparedness exercises at the Australian Animal Health Laboratory. In 2013, the ELISA was transferred to Australian state laboratories as part of a Laboratories-for-Emergency-Animal-Disease-Diagnosis-and-Response exercise to establish specificity estimates for Australian livestock species and a proficiency test round was performed. Initial estimates for robustness and reproducibility were satisfactory and the 3ABC ELISA has been included as an approved test by the Sub-committee on Animal Health Laboratory Standards.[25]

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