Comparative evaluation of four competitive/blocking ELISAs for the detection of influenza A antibodies in horses

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ABSTRACT

New Zealand is free from equine influenza and has never experienced an incursion in its horse population. As part of New Zealand’s preparedness to an incursion of an exotic animal disease, it was considered necessary to select the most accurate test for equine influenza (EI) from the array of those available. Four readily available blocking/competitive enzyme-linked immunosorbent assays (ELISA), originally developed and marketed for the detection of antibodies against the avian influenza virus, were evaluated using serum samples from New Zealand non-infected, non-vaccinated horses (n = 365), and Australian field infected (n = 99) and experimentally infected horses (n = 3). Diagnostic specificities (DSP) and diagnostic sensitivities (DSE) were determined as follows: ELISA-1 = 98.1%/99.0%; ELISA-2 = 90.1%/99.0%; ELISA-3 = 98.1%/96.0%; ELISA-4 = 95.3%/99.0%. For ELISA-1, DSP and DSE results were comparable to previously published data on a larger sample number from Australian horses (Sergeant et al., 2009).

Receiver operating characteristics (ROC) and frequency histogram analysis were also performed. The area under the curve (AUC) ranged from 0.996 to 0.979, with ELISA-1 possessing the highest AUC, followed by ELISA-2, ELISA-4 and ELISA-3. Separation of the negative and the positive serum panel was best for ELISA-4, followed by ELISA-2, ELISA-1 and ELISA-3. In three experimentally infected horses, sero-positivity was detected between 7 and 9 days post-infection, with ELISA-4 being most sensitive, followed by ELISA-1, ELISA-2 and ELISA-3. Overall, the four ELISAs performed well in this evaluation but some differences were observed.

1. Introduction

Equine Influenza is a widely distributed acute respiratory disease of equidae and is caused by two distinct subtypes (H7N7 and H3N8) of the influenza A virus (Myers and Wilson, 2006; Daly and Mumford, 2008). The virus is present in most countries with large equine populations. In recent years it has been introduced into South Africa, Japan and Australia (Toulemonde et al., 2005; Yamanaka et al., 2008; Cowled et al., 2009). To date, only Iceland and New Zealand have remained free from the infection (Van Maanen and Cullinane, 2002; Cowled et al., 2009).

A large outbreak of EI, typified by an H3N8 influenza A virus subtype (influenza A/equine/Sydney/2888-8/2007) and associated with imported horses, occurred in August 2007 in Australia, which had previously been free from EI...
This was the largest animal disease emergency in Australia’s history with more than 10,000 premises affected and an estimated 76,000 horses infected (Anon., 2009). The virus was completely eradicated and country freedom declared on 30 June 2008. Accurate diagnosis of infected horses was critical during the eradication phase. Real-time polymerase chain reaction (RT-PCR) and a blocking ELISA, initially developed for the detection of avian influenza antibodies in commercial poultry at the Australian Animal Health Laboratory (AAHL), were the primary diagnostic tools used during the incursion response and post-outbreak testing. By the end of 2007, about 53,000 RT-PCR and more than 60,000 ELISA tests had been performed. Retrospectively, with the field testing data available from the EI epidemic in Australia, this ELISA had been evaluated with a DSE of 99.2% (n = 475, PCR confirmed infected horses) and a DSP of 96.7% (n = 1323, horses from un-infected zones in New South Wales, Australia). (Sergeant et al., 2009).

The AAHL ELISA had been extensively used in the Australian EI outbreak specifically for proving of absence of EI in certain regions, for distinguishing between horses vaccinated with the canarypox vectored vaccine and infected horses, and for detecting previous infection after the virus had been cleared from the animal, i.e. when infection could not be picked up anymore by PCR. In New Zealand, the ELISA would be used for similar purposes. New Zealand is a country free from EI but with close links and horse travel to and from Australia and other countries where EI outbreaks have occurred. Consequently, it is important that adequate preparations for a potential EI incursion are made. While the diagnostic performance of the AAHL ELISA was known for Australian horses, for other commercial influenza A antibody detection ELISAs none or very limited data were available for horses. This paper reports on the specificity of four blocking/competitive ELISAs in non-infected New Zealand horses, and the sensitivity from field and experimentally infected Australian horses. ROC analysis was also performed.

2. Materials and methods

2.1. Serum samples

Panel-1 (influenza-naïve horses) consisted of a total of 365 blood samples from horses within New Zealand, unexposed by vaccination or natural disease (disease free because EI is not present in the country). Purposive sampling methods were used to minimise costs and allow active collection of sera from horses known to be unexposed to equine influenza antigen. Horses were only rejected for inclusion in the study if they had travelled overseas or had been vaccinated for equine influenza. Collection was performed during various horse events and by private practitioners between 29 November 2008 and 20 March 2009. Samples were obtained from horses with a wide geographical distribution within New Zealand and also from many different horse breeds. Nevertheless, this panel has to be considered a “convenience” panel as no pre-specified sampling plan was used, other than what has been said above. No pre-testing was done and no samples were excluded. Blood collection was covered by the Animal Ethics Approval 11664, AgResearch animal Ethics Committee, Palmerston North, New Zealand.

Panel 2 (naturally infected horses) consisted of 99 serum samples from non-vaccinated Australian horses that had been infected during the 2007 EI outbreak and was used as a positive control panel. This was a subsample of the positive panel described by Sergeant et al. (2009). These horses were considered infected on the basis of a positive result in an influenza A group reactive real time RT-PCR test (qRT-PCR) (Heine et al., 2007). The serum samples for ELISA testing were taken from these horses at least 10 days after the positive qRT-PCR. The RT-PCR was performed at the Elizabeth Macarthur Agriculture Institute, Camden, NSW, Australia.

Panel-3 (experimentally infected horses) consisted of 43 serum samples taken from three horses experimentally infected with the EI virus (A/equine/Sydney/2888-8/2007) at the AAHL (Foord et al., 2009). This panel was used to compare the ELISAs in their ability to detect seroconversion post-infection (analytical sensitivity).

2.2. Serological tests

ELISA-1 is an antibody detection ELISA that was used in a blocking format (bELISA), originally developed to detect antibodies to avian influenza at the CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, VIC, Australia (Sergeant et al., 2009). In a blocking ELISA, serum samples are incubated in the ELISA plates first, where specific antibodies will bind to the antigen, and then after washing, the anti-antigen-conjugate is incubated. In a competitive ELISA, serum samples are incubated together with the anti-antigen-conjugate in one step. Testing of serum panels-1 and -2 by ELISA-1 was performed at the Elizabeth Macarthur Agriculture Institute, Camden, NSW, Australia. Testing of panel-3 by ELISA-1 and in the haemagglutination inhibition (HI) test was performed at the AAHL.

ELISA-2 is the Avian Influenza Virus Antibody ELISA from Anigen Animal Genetics Inc., Woncheon-dong, Korea, a competitive ELISA (cELISA). ELISA-3 is the FlockChek™ AI MultiS-Screen Antibody Test Kit from IDEXX Laboratories, Westbrook, Maine, a bELISA. ELISA-4 is the ID Screen™ Influenza A Antibody Competition ELISA kit from ID-Vet, Montpellier, France, which works in a bELISA format even its name suggests a competitive format. Testing of the three serum panels in ELISA-2, -3 and -4 was performed at the Investigation and Diagnostic Centre, Animal Health Laboratory, Upper Hutt, New Zealand.

Each of the four ELISAs uses antigens based on the influenza A virus nucleoprotein and a monoclonal anti-nucleoprotein horseradish peroxidase conjugated antibody. Not many details were available from the manufacturers on the antigens used: ELISA-1: recombinant influenza A virus nucleoprotein antigen expressed in E. coli; ELISA-2: recombinant nucleoprotein antigen; ELISA-3 and ELISA-4: influenza A virus nucleoprotein. The ELISAs were performed according to the manufacturers’ instructions. On completion of the immune reactions, optical
In accordance with the manufacturers’ instructions, percent inhibition (PI) was calculated: \( PI = 100 \times (1 - \frac{OD_{sample}}{OD_{negative \ control}}) \). Samples with \( PI \leq 50% \) were considered negative for each ELISA. Samples with \( PI > 50% \) were considered positive. Samples with \( PI \) between 50% and 60% in ELISA-1, and between 50% and 55% in ELISA-4, were considered suspicious. For evaluation of the ELISAs, suspicious samples were counted as positives.

The HI test was performed as described in Daly and Mumford (2008) and Oh et al. (2009). Briefly, serum samples were inactivated at 56°C for 30 min, then 25 μl of a twofold serial dilution of the serum samples in phosphate-buffered saline were incubated at room temperature for 1 h with an equal volume of 4 haemagglutination units of the virus, followed by the addition of 50 μl of erythrocytes. The reciprocal of the highest dilution of the antibody showing no haemagglutination was noted as the HI titre.

### 2.3. Statistical methods

Frequency histograms for non-infected (panel-1) and infected (panel-2) horses were generated for each ELISA to display the distribution of PI results in both classes of horses with the aid of Excel worksheets. Median PI values for non-infected and infected populations were obtained from the ROC analysis (see below). Separation values between non-infected and infected population was calculated by subtracting the median PI value for the non-infected population from the median PI value of the infected population for each ELISA.

Statistical analyses were undertaken using Epitools, a free online site offering a variety of epidemiological analytical tools (Sergeant, 2009).

Specificity (proportion of non-infected horses that tested negative, panel-1) and sensitivity (proportion of infected horses that tested positive, panel-2) for each ELISA were calculated by using the tool: test evaluation against a gold standard (and ROC analysis, see below) with confidence intervals calculated as exact binomial limits (Sergeant, 2009).

ROC analysis for every ELISA was performed over a range of possible cut-off points, based on ELISA PI values for the non-infected New Zealand horses (panel-1) and the infected Australian horses (panel-3) by using the tool: calculate test sensitivity and specificity and ROC curves (Sergeant, 2009).

### 3. Results

ELISA DSPs ranged from 90.1% to 98.1% and DSEs from 96.0% to 99.0% (Table 1). In ELISA-2, at total of 36 samples from panel-1 (non-infected, non-vaccinated New Zealand horses) tested positive. From these 36 false positive samples, 17 samples tested positive in ELISA-4, and from these 17 samples, seven tested positive in ELISA-1 and ELISA-3. An 18th sample tested suspicious in ELISA-4 but was negative in the other three ELISAs.

In panel-2 (Australian field infected horses), ELISA-1, ELISA-2 and ELISA-4 tested one sample as negative. ELISA-3 tested four samples with negative results. The negative sample from ELISA-1 was identical to one of the negative samples in ELISA-3. The negative sample in ELISA-2 and ELISA-4 were identical, were different to the negative sample in ELISA-1, and tested also negative in ELISA-3.

Frequency histograms derived from PI values for panel-1 and panel-2 samples are shown in Fig. 1 and calculated separation data for the non-infected and infected group in Table 2. For each of the four ELISAs, PIs from the infected and non-infected panel appear to be well separated. ELISA-4 shows the best separation, followed by ELISA-2 and ELISA-1, and ELISA-3 with the lowest separation. For ELISA-2, PIs from the infected panel are concentrated at the high PI end of the spectrum, falling almost exclusively into the 95–100% PI bracket.

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Diagnostic specificity (n = 365)</th>
<th></th>
<th>Diagnostic sensitivity (n = 99)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DSP Lower 95% CL Upper 95% CL</td>
<td>DSP Lower 95% CL Upper 95% CL</td>
<td></td>
</tr>
<tr>
<td>ELISA-1</td>
<td>0.981 0.961 0.992</td>
<td>0.990 0.945 1.000</td>
<td></td>
</tr>
<tr>
<td>ELISA-2</td>
<td>0.901 0.866 0.930</td>
<td>0.990 0.945 1.000</td>
<td></td>
</tr>
<tr>
<td>ELISA-3</td>
<td>0.981 0.961 0.992</td>
<td>0.960 0.900 0.989</td>
<td></td>
</tr>
<tr>
<td>ELISA-4</td>
<td>0.995 0.927 0.973</td>
<td>0.990 0.945 1.000</td>
<td></td>
</tr>
</tbody>
</table>

DSP = diagnostic specificity; DSE = diagnostic sensitivity; \( n \) = number of samples (animals); CL = exact binomial confidence limit.

### Table 2

<table>
<thead>
<tr>
<th>ELISA type</th>
<th>Median PI</th>
<th>Difference between median PIs</th>
<th>Area under curve</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-infected</td>
<td>Infected</td>
<td></td>
</tr>
<tr>
<td>ELISA-1</td>
<td>4.0</td>
<td>88.2</td>
<td>84.2</td>
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<tr>
<td>ELISA-2</td>
<td>10.5</td>
<td>99.3</td>
<td>88.8</td>
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<tr>
<td>ELISA-3</td>
<td>3.9</td>
<td>81.5</td>
<td>77.6</td>
</tr>
<tr>
<td>ELISA-4</td>
<td>3.3</td>
<td>94.2</td>
<td>90.9</td>
</tr>
</tbody>
</table>

ROC = receiver operating characteristics; PI = percent inhibition; AUC = area under curve; CL = exact binomial confidence limit.

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ROC analysis results using panel-1 and panel-2 are shown in Table 2 and Fig. 2. In the ROC analysis graphs (Fig. 2), each ELISA shows a curve that one would expect from a well performing test method, i.e. the curves run very close to the left and upper boundary. Measuring the AUC showed that there were some differences in ELISA performance with ELISA-1 covering the biggest area, followed by ELISA-2, ELISA-4 and ELISA-3.

Some differences in the serological response were seen in experimentally infected horses for the HI test and the four ELISAs (Fig. 3). The HI test identified the horses as infected 9 days post-infection (p.i.) when using a fourfold increase in titre as criteria for recent infection (Daly and Mumford, 2008). ELISA-4 was most sensitive (7 days p.i.), followed by ELISA-1 and ELISA-2 (8 days p.i.), and ELISA-3, which detected sero-conversion least (9 days p.i.).

4. Discussion

This study was undertaken to evaluate the performance of four competitive/blocking ELISAs for the detection of influenza A antibodies in horses in order to identify which ELISA should be preferably used in the event of an EI incursion in New Zealand. These ELISAs were chosen because they were readily available. ELISA-1 had been used during the 2007 EI outbreak in Australia and had been shown to be highly accurate (Sergeant et al., 2009). The other three ELISAs are commercially available ELISAs that had been developed and evaluated for avian influenza testing in birds. Evaluation data for equine influenza were not available for the latter ELISAs or only to a very limited extend for one of the test kits (ELISA-2) by the manufacturer, stating a DSE of 100% based on 63 horses.

Having accurate values for DSE and DSP available is important in the intended use of a test method. A test with a higher DSE should be applied early in a response where it is important to identify all new clusters of infection. DSP increases in importance where eradication is an achievable objective, as it is important to explain all positive results. ELISA-1 showed the highest DSE of 99.0%, which was close to the published DSE of 99.2%, even though the number of horses tested here was about 1/5th of the...
published evaluation (Sergeant et al., 2009). The reason for this good agreement may be that both populations were derived from the same animal pool, i.e. Australian field infected horses from the 2007 EI outbreak.

The DSP for ELISA-1 was also one of the highest of the four ELISAs at 98.1% and slightly better than the published value of 96.7% even so the number of samples used in our study was about 1/3 of the samples applied in the published study (Sergeant et al., 2009). The lower DSP of the Australian study may be explained by differences in the sampling procedures. In New Zealand, every attempt had been made to exclude vaccinated horses from being sampled. This was achieved by collecting at horse events where only horses were present that never had travelled outside the country, as was confirmed by their owners. Only horses leaving New Zealand are required to be vaccinated for EI. However, in the Australian study, also every attempt had been made to exclude horses that had been vaccinated with vaccines that could cause an antibody response to viral nucleoprotein (Sergeant et al., 2009). Other, unrecognised reasons for the slight discrepancy in DSP values may exist.

For the other three ELISAs either the DSP or DSE could be as high as for ELISA-1 but if the DSE was high, the DSP was lower and vice versa. In this respect, these ELISA behaved like typical serological test methods, i.e. sensitivity and specificity changed as a function of the cut-off in an inverse manner.

False positive samples in ELISAs with higher DSPs generally tested false positive in ELISAs with lower DSPs as well, which means that the same samples tested false positive in every ELISA. This indicates that false positivity has the same underlying reasons in each of the EI ELISAs evaluated. This could be due to the very similar design of the ELISAs, using the influenza virus nucleoprotein as antigen and a monoclonal antibody (Mab) with specificity for one epitope to compete with serum antibodies. The Mabs’ epitope specificities and binding characteristics in the various ELISAs were not available and could therefore not be compared. Furthermore, while two ELISA manufacturers state that they use recombinant antigens, for the other two ELISAs the manufacturers’ only statement was that nucleoprotein antigen had been used. There may be differences in antigen preparations as well as in the Mabs used which could have contributed to differences observed for the evaluation parameters.

The similar characteristic of the four ELISAs in giving false positive and false negative results makes them unsuitable for use in serial testing, i.e. to re-test an animal that had been positive in one ELISA with another ELISA (Gardner et al., 2000). Unlike in other studies, for example in a study where a competitive and an indirect ELISA were conditionally independent enough to increase overall testing specificity when used in series (Kittelberger et al., 2008), this is not the case here for the influenza ELISAs.

Fig. 2. Receiver operating characteristic (ROC) analysis of four ELISAs using panels of horse serum from non-infected, non-vaccinated New Zealand horses (n = 365) and field infected Australian horses (n = 99).
We have to be aware that in an EI outbreak situation, vaccinated horses may test positive by the ELISA kits evaluated. Currently, horses that travel overseas are vaccinated in New Zealand, depending on the country they are travelling to. The vaccines approved for this purpose will cause an antibody response detectable in the ELISAs evaluated here. In case of an EI incursion, differentiation between vaccination and infection is required and can be achieved by using a recombinant canarypox vaccine, as was the case during the Australian EI outbreak (Cowled et al., 2009).

5. Conclusions

The four competitive/blocking ELISAs evaluated in this study performed well but showed a certain degree of difference in their performance. ELISA-1 is the test method that should be used if large scale testing of horses during an EI outbreak would be necessary. ELISA-1 possessed high DSP and DSE, exhibited a good separation of results from infected and non-infected animal groups, showed good receiver operator characteristics, and was able to detect infection early in experimentally infected animals.

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Fig. 3. Serological response in three horses (panel-3) experimentally infected with the influenza virus subtype A/equine/Sydney/2888-8/2007. Horses were infected at day 0. PI = percent inhibition in ELISA; HI = haemagglutination inhibition. - - - - - cut-off for ELISA. Note: for ELISA-2, -3, and -4, bloods were not taken at days 1–4 post-infection.
freely available or at reduced costs: IDEXX Laboratories, Anigen Animal Genetics Inc., and ID-Vet Laboratories.

References


