Combining two serological assays optimises sensitivity and specificity for the identification of Streptococcus equi subsp. equi exposure

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ABSTRACT

The detection of anti-Streptococcus equi antibodies in the blood serum of horses can assist with the identification of apparently healthy persistently infected carriers and the prevention of strangles outbreaks. The aim of the current study was to use genome sequencing data to develop an indirect enzyme linked immunosorbent assay (iELISA) that targets two S. equi-specific protein fragments. The sensitivity and specificity of the antigen A and antigen C iELISAs were compared to an SeM-based iELISA marketed by IDvet – diagnostic Vétérinaire (IDvet). Individually, each assay compromised specificity in order to achieve sufficient sensitivity (SeM iELISA had a sensitivity of 89.9%, but a specificity of only 77.0%) or sensitivity to achieve high specificity. However, combining the results of the antigen A and antigen C iELISAs permitted optimisation of both sensitivity (93.3%) and specificity (99.3%), providing a robust assay for the identification of horses exposed to S. equi.

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Introduction

Strangles, caused by Streptococcus equi subspecies equi (S. equi), is the most frequently diagnosed infectious disease of horses worldwide with over 600 outbreaks identified each year in the UK alone (Parkinson et al., 2011). The disease is characterised by abscessation of the lymph nodes of the head and neck. Abscesses formed in the retropharyngeal lymph nodes usually rupture into the mucous pouches, which drain via the nostrils leading to the classical mucopurulent nasal discharge associated with strangles. However, the purulent material in the guttural pouches of a proportion of animals fails to drain completely, enabling live S. equi to persist in horses that have recovered from the acute disease for up to several years in the absence of obvious clinical signs (Newton et al., 1997). S. equi periodically sheds from these persistently infected carrier horses, allowing transmission to naïve individuals and new outbreaks of disease. The persistence of S. equi in the guttural pouches of horses is associated with follicular hyperplasia, suggesting that it may be possible to detect a measurable antibody response (Waller and Jolley, 2007).

The immunogenic SeM protein binds fibrinogen, and IgG4 and IgG7 sub-classes resulting in an anti-phagocytic activity (Boschwitz and Timoney, 1994; Meehan et al., 1998, 2001; Lewis et al., 2008). Although mice vaccinated with SeM were protected from intraperitoneal challenge (Meehan et al., 1998), no protection was conferred to horses (Sheoran et al., 2002). Vaccination with SeM-containing vaccines has been linked with complications such as purpura haemorrhagica (Pusterla et al., 2003) and the perceived risk of this often fatal complication led to the development of a SeM-based indirect enzyme-linked immunosorbent assay (iELISA) for the identification of horses with high anti-SeM antibody levels pre-vaccination with SeM-containing vaccines. This iELISA has subsequently been exploited for the identification of horses infected with S. equi, with high antibody titres being suggested to indicate disseminated disease, known as bastard strangles, although no sensitivity or specificity data have been published to date (Sweeney et al., 2005).

The SeM protein was also thought to be restricted to S. equi (Galan and Timoney, 1988; Timoney et al., 1997), but a homologue, SzM, in S. zooepidemicus was identified that shares near identity with SeM across the C-terminal two-thirds of this protein (Kelly et al., 2006). Pre-incubating sera with heat-killed S. zooepidemicus can remove cross-reactive antibodies to SzM prior to the detection of SeM-specific antibody responses (Davison et al., 2008). Such a process, although successful in reducing assay background, has not
been implemented in current SeM-based assays, which continue to be based on full length SeM despite the potential for the reporting of false positive results.\textsuperscript{1,2}

We exploited the \textit{S. equi} strain 4047 (Se4047) and \textit{S. zooepidemicus} strain H70 (SzH70) genome sequences to identify fragments of surface exposed or secreted proteins that were restricted to \textit{S. equi} (Holden et al., 2009). We report here the application of N-terminal recombinant protein fragments of SEQ2190 (antigen A) and SeM (antigen C), for the development of an iELISA for the detection of a \textit{S. equi}-specific antibody response. The performance of the antigen A and antigen C iELISAs relative to a whole SeM-based iELISA for the identification of horses exposed to \textit{S. equi} was determined.

Materials and methods

Production of recombinant antigen A and antigen C

The 5’ regions of the SEQ2190 and SeM genes that are restricted to \textit{S. equi} were amplified by PCR from Se4047 chromosomal DNA with Phusion polymerase (NEB) and the primers 5’ 595sequiunuc (CCCGGATCTTAAAGAAGGCGATGTCACAAAAGG) and 3’ 595sequiunuc (AAGAGATCTTGGTTCTACCGTACCTGTC) or 5’ MPEPI (AAGAGATCTTAAATCTCAGAACAGAAGT) and 3’ MPEPI (TTTGGAATCTTACGCTTTAGCTCTTCC), respectively (Baumfi/EcorI restriction sites are underlined). The PCR products were cloned into the BamHI/EcoRI sites of the pGEX-3X vector (GE Healthcare). The resulting constructs, pGEX-2190 and pGEX-mpep1, contain N-terminal fusions of antigen A and antigen C to a glutathione S-transferase (GST) tag driven by a tac promoter. DH10B E. coli cells harbouring each plasmid were grown at 37 °C in 2 × YT containing 50 μg/mL ampicillin. Once the cells reached an optical density at 600 nm of 0.6, 1 mM isopropyl-β-D-thiogalactopyranoside was added to the medium and the cultures were incubated for 4 h at 28 °C. Cells were harvested and lysed by digestion with lysozyme for 1 h at 4 °C followed by sonication, and the GST-antigen A and GST-antigen C fusions purified over glutathione Sepharose 4B beads according to the supplier’s protocol (GE Healthcare).

Serum samples used for assay comparison

A serum set from 89 horses involved in outbreaks of strangles in the UK was used to determine assay sensitivity. Nasopharyngeal or guttural pouch samples from all of these horses were culture positive for \textit{S. equi} at or shortly before the time of sampling and all were regarded as ‘true positive’ samples.

A serum set from 139 Icelandic horses was used to determine assay specificity. The Icelandic horse population is geographically isolated with virtually no import of horses for over 1000 years. Consequently, Icelandic horses have remained free from most common contagious diseases including equine influenza, equine rhinopneumonitis, equine herpesvirus (EHV-1), equine viral arteritis (EVA) and strangles. The Icelandic horse population is geographically isolated with virtually no import of horses for over 1000 years. Consequently, Icelandic horses have remained free from most common contagious diseases including equine influenza, equine rhinopneumonitis, equine herpesvirus (EHV-1), equine viral arteritis (EVA) and strangles.

\textbf{iELISA protocol}

Ninety-six well Immulon 2HB Flat bottom microtitre plates were coated overnight at 4 °C with 200 ng of antigen A or antigen C per well. Plates were washed with phosphate buffered saline containing 0.05% Tween 20 (PBST) and blocked with PBST containing 1% non-fat milk (PBSTN) for 1 h at 37 °C. Plates were washed with PBST and 100 μL of test or control serum diluted 1/800 in PBSTN was added to the appropriate well for 1 h at 37 °C. Assays were run in duplicate. Plates were washed with PBST and 100 μL of peroxidase-conjugated goat anti-horse IgG (Kirkegaard and Perry Laboratories) diluted 1/600 in PBSTN was added to each well and the plates incubated for 1 h at 37 °C. Plates were washed with PBST, 100 μL of SureBlue re-serve peroxidase substrate 3,3’,5,5’-tetramethylbenzidine (Kirkegaard and Perry Laboratories) was added and the plate incubated for 10 min at 21 °C. 100 μL of 0.18 M sulphuric acid was added and the optical density at 450 nm (OD\textsubscript{450}) measured. An OD\textsubscript{450} of >0.5 was considered to be a positive result for antigen A or antigen C iELISAs and for the combined assay a sample was considered to be positive if either assay generated a positive result.

\textbf{SeM iELISA protocol}

Ninety-six well plates that were pre-coated with full-length recombinant SeM were purchased from iDvet (Streptos ver 0212 GB). The iELISA assay was conducted according to manufacturer’s instructions. Briefly, test and control sera were diluted 1/200 in the dilution buffer provided. 100 μL of the appropriate diluted serum was added to each well and the plate incubated for 1 h at 37 °C. Assays were run in duplicate. Plates were washed, 100 μL of 1:1 conjugate added and incubated for 1 h at 37 °C. Plates were washed, 100 μL of substrate solution added and incubated for 10 min at 21 °C in the dark. One hundred microlitres of stop solution were added to each well and the OD\textsubscript{450} measured. Positive control sera generated an OD\textsubscript{450} of >0.350 and the ratio of the OD\textsubscript{450} of positive to negative control sera was greater than 3, in agreement with manufacturer’s guidelines. The percentage of sample OD\textsubscript{450} relative to the positive control for each plate was calculated according to manufacturer’s instructions. A percentage of > 100 was considered positive.

Statistical analyses

To compare the iELISA results in a graphical format, the mean% assay result relative to the cut-off was calculated for the antigen A and antigen C assays by dividing the iELISA result by 0.5 (the assay cut-off) and multiplying by 100.

The ability of the two tests to confirm exposure to \textit{S. equi} was investigated by cross tabulating test result classification (positive or negative) against the true exposure status (gold standard diagnosis) based on the origin of the samples (bacteriologically confirmed UK exposure for positives and resident Icelandic status for negatives) and calculating sensitivity and specificity values based on the following definitions. Sensitivity is represented by the proportion of presumed true positives that are identified as positive by the test being evaluated, with (100% minus specificity) representing the proportion of false negative results detected by the test. Specificity is represented by the proportion of presumed true negatives that are identified as negative by the test being evaluated, with (100% minus specificity) representing the proportion of false positive results detected by the test.

Data were also analysed using Student’s t test and Receiver Operating Characteristics (ROC) in Stata12 software to assess sensitivity and specificity estimates for all % relative to assay positive control’ breakpoints within the data applied against the gold standard diagnosis.

Results

All three iELISA assays generated significantly higher results with sera from strangles outbreaks than from Icelandic resident horses (P < 0.0001 for all three assays; Fig. 1). At the manufacturer’s cut-offs (100% relative to assay positive control), the SeM iELISA correctly identified 80/89 positive and 107/139 negative samples giving a sensitivity of 89.9% and specificity of 77.0%, respectively (Table 1). The results for each serum sample across all of the iELISAs are presented in Table S1 (Appendix A). ROC analysis identified that a breakpoint of 130% relative to the assay control correctly identified 72/89 positive and 135/139 negative samples generating an optimal assay sensitivity and specificity of 80.9% and 97.1%, respectively with this sample set (Fig. 2).

The antigen A iELISA correctly identified 66/89 positive and 138/139 negative samples giving a sensitivity of 74.2% and specificity of 99.3% (Table 1). The antigen C iELISA was less sensitive.


\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Comparison of the three iELISA assays. The mean% assay result relative to the cut-off for sera from Icelandic residents (n = 139) or from strangles outbreaks (n = 89) was calculated for each of the iELISAs. Error bars show the 95% confidence interval. The Icelandic sera iELISA results for all of the assays were significantly different to those from strangles outbreaks (P < 0.0001).}
\end{figure}
The identification of horses that possess a specific antibody response to S. equi in the absence of clinical signs of disease using appropriate iELISAs can direct further sampling strategies towards the identification and treatment of persistently infected carriers and the eradication of S. equi infection. However, it is essential that these assays have excellent sensitivity and specificity to minimise re-sampling and the associated costs. S. equi is endemic in the UK horse population and even apparently healthy herds may contain persistently infected carriers or exposed horses that would generate genuinely positive iELISA results. However, there have been no outbreaks of strangles in Iceland and no import of horses is permitted, making this an ideal negative population for the quantification of the specificity of strangles diagnostic assays.

Although the IDvet SeM-based iELISA assay was almost as sensitive as the combined antigen A + antigen C iELISA (89.9% cf. 93.3%), it had a poorer specificity, falsely identifying 23.0% of samples from Icelandic horses as positive for S. equi. It is considered that this level of misclassification would incur an unacceptable level of additional intervention, which would be likely to affect long term compliance with any screening programme based on such a test. Increasing the breakpoint of the SeM iELISA to 130% increased assay specificity to 97.1%, but at the expense of reduced sensitivity, such that almost 1/5 true positive horses would be incorrectly classified.

The misclassification of horses based on the SeM iELISA was not unexpected given that a homologue of SeM, SzM, which shares near identity across its C-terminal two thirds, is encoded by S. zooepidemicus (Kelly et al., 2006). Cross-reactivity of anti-SzM antibodies, produced following infection of Icelandic horses with S. zooepidemicus, with SeM in the IDvet assay provides one explanation for false positive results and the higher mean Icelandic sera result for the IDvet assay observed in Fig. 1. The selection of protein fragments that are restricted to S. equi on the basis of genome content generated a highly sensitive and specific iELISA. The use of both antigen A and antigen C iELISA resulted benefitted clinical interpretation by raising sensitivity from 74.2% and 59.6%, respectively to 93.3%. In the population of Icelandic resident horses included in this study, the specificity of the combined antigen A and antigen C iELISA was 99.3%, with only one of 139 negative horses misclassified.

The international population of S. zooepidemicus is highly diverse (Webb et al., 2008) with over 300 different multilocus sequence types identified to date. Although S. zooepidemicus is commonly isolated from the Icelandic horse population (S. Björnsdóttir et al., unpublished data), the geographical isolation of these horses suggests that the diversity of the Icelandic population of S. zooepidemicus strains is likely to be lower than in populations of horses.

Table 1

<table>
<thead>
<tr>
<th>Sera from S. equi positive horses</th>
<th>Sera from Icelandic resident horses</th>
<th>Total n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SeM</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>80 (89.9%)</td>
<td>32</td>
</tr>
<tr>
<td>Negative</td>
<td>9</td>
<td>107 (77.0%)</td>
</tr>
<tr>
<td>Total</td>
<td>89</td>
<td>139</td>
</tr>
<tr>
<td><strong>Antigen A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>66 (74.2%)</td>
<td>1</td>
</tr>
<tr>
<td>Negative</td>
<td>23</td>
<td>138 (99.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>89</td>
<td>139</td>
</tr>
<tr>
<td><strong>Antigen C</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>53 (59.6%)</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>36</td>
<td>139 (100%)</td>
</tr>
<tr>
<td>Total</td>
<td>89</td>
<td>139</td>
</tr>
<tr>
<td><strong>Antigen A and C</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>83 (93.3%)</td>
<td>1</td>
</tr>
<tr>
<td>Negative</td>
<td>6</td>
<td>138 (99.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>89</td>
<td>139</td>
</tr>
</tbody>
</table>

Fig. 2. Receiver Operator Characteristics curve for iELISA data generated using Stata12 software to assess sensitivity and specificity estimates for all % relative to assay positive control cut-off points within the data applied against the gold standard diagnosis. Sensitivity% is represented by the proportion of presumed true positives that are identified as positive by the test being evaluated. Specificity% is represented by the proportion of presumed true negatives that are identified as negative by the test being evaluated, with (100% minus specificity%) representing the proportion of false positive results detected by the test.

Table 1: Ability of the antigen A + antigen C and IDvet SeM serological tests to confirm exposure to S. equi.

**Discussion**

The identification of horses that possess a specific antibody response to S. equi in the absence of clinical signs of disease using appropriate iELISAs can direct further sampling strategies towards the identification and treatment of persistently infected carriers...
without import restrictions. Therefore, the iELISA specificity figures presented in this study could decline in horse populations infected with a more diverse array of \textit{S. zooepidemicus} that may express cross-reactive epitopes. Although beyond the scope of the current study, it is known that horses that have recovered from strangles are also likely to generate positive iELISA test results for several months even if they have eliminated \textit{S. equi} infection. Following the resolution of clinical signs the screening of all non-affected horses on the premises by iELISA enables the identification of horses that were inadvertently exposed to \textit{S. equi} during the outbreak, or may be unknown persistently infected carriers. The identity of any persistently infected carriers can then be confirmed by qPCR (Webb et al., 2012) on testing three consecutive nasal swabs at weekly intervals or a guttural pouch lavage from all convalescent horses and those unaffected animals that were iELISA positive. Such a post-outbreak process enables the elimination of \textit{S. equi} from the resident population and the prevention of recurrent cases of strangles.

Conclusions

The antigen A + antigen C combined iELISA is currently the best available serological screening assay for evaluating horses’ exposure to \textit{S. equi} with adequately high levels of sensitivity and specificity for use in eradication and health screening programmes.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tvjl.2013.01.033.

References


