Decreased Clinical Severity of Strangles in Weanlings Associated with Restricted Seroconversion to Optimized Streptococcus equi ssp equi Assays

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Streptococcus equi ssp. equi (hereafter referred to as S. equi) is an obligate pathogen, causing disease only in horses. Characteristic clinical signs include fever, accompanied by copious purulent nasal discharge, abscessation and rupture of lymph nodes in the head and neck region, followed by recovery over several weeks.1 The exudate from nasal cavity and draining lymph nodes of clinically ill horses is critical for effective spread of the disease to immunologically naive stall mates. Outbreaks in young horses are reportedly the most serious clinically,1 with morbidity of approximately 90% in infections of immunologically naive foals2 and 100% in experimental infection.3,4 A detailed plan for infection control and containment at the onset of strangles outbreaks in larger groups of horses involves dividing horses on a strangles-affected premise into 3 color categories: red, being those with clinical signs of strangles; amber, those horses lacking clinical signs but in contact with strangles cases; and green, those animals with neither clinical signs nor contact.5 In theory, this regimen is a clearly defined plan to effectively isolate infected from uninfected horses and contains the infection until it runs its course. The rectal temperatures of horses in the amber group are monitored regularly and those that develop fever are moved to the red group. If no fever occurs in horses in the amber group over the course of the outbreak, it is assumed they have not been infected and need not undergo the microbiologic testing that is advised for the infected (red) group. However, all horses should be assessed serologically for exposure before releasing the yard from isolation. Although these guidelines represent a robust biosecurity protocol, routine access to optimal serologic tools, the decision of when to release the yard from isolation rests on the microbiological testing. Questions

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faced by the consulting veterinarian are whether or not all horses must undergo the expense of microbiological screening, in particular, those that remain clinically normal and lack clear evidence of contact with diseased animals.

Our aim was to monitor a natural outbreak of strangles in a large group of serologically naïve weanling horses to determine whether clinical or laboratory results obtained from routine health checks could predict individuals in which S. equi persisted as a silent carrier status.

Materials and Methods

Animals

One-hundred and twelve recently weaned warmblood foals, 7–10 months of age and consisting of 51 males and 61 females, were housed together based on sex in separate paddocks with wind shelters. Nose-to-nose contact was possible between paddocks, and all horses on the farm were under daily observations by the farm staff and had regular veterinary observation and care, including periodic blood sampling.

Clinical Sampling and Sample Analysis

Shortly after weaning in early autumn of 2014, several individuals in the group developed clinical signs suggestive of acute strangles (see “clinical scoring” below) which subsequently spread throughout the group. No biosecurity measures within these groups were implemented, and the outbreak was allowed to run its course to clinical recovery of the groups. When the outbreak appeared to peak in clinical severity in mid-November 2014, all animals in the cohort underwent full clinical examinations, followed by obtaining blood samples for total white blood cell counts as well as nasal swab sampling approximately 15 cm caudal to the nares using amies-charcoal swabs for culture and qPCR for S. equi and Streptococcus equi ssp. zooepidemicus (hereafter referred to as S. zooepidemicus). Vinyl gloves were used for all nasal swabbing with gloves discarded and changed between each horse examined. Detailed clinical examinations or clinical examinations of the head and neck regions, and blood sampling for white blood cell counts were repeated on the entire group subsequently until the outbreak had resolved, with final sampling 19 weeks after the initial sampling (Fig 1). Serum samples from this group of horses obtained 6 and 3 weeks before nasal swabbing as well as during and up to the 19 weeks after the peak of the outbreak were stored at –20°C for subsequent serological analysis by an optimized ELISA that targets both antigen A (SEQ_2190) and antigen C (SeM) of S. equi. None of the clinically affected horses was treated with antibiotics.

Clinical Scoring

Individuals were assigned a clinical score as adapted as previously described based on clinical observations recorded on each animal during each of the specific clinical examination and sampling occasions. As shown in Table 1, the clinical score was designed to identify and assemble clinical abnormalities suggestive of acute strangles and included the presence of fever, defined as a rectal temperature >38.2°C, nasal discharge, lymph node swelling, abscessation or both and at least moderate leukocytosis (≥14.0 × 10³μL⁻¹). Those assigning clinical variables included in the final score were unaware of nasal swab and serologic results.

For the sake of this investigation, a total clinical score of ≥4 was deemed sufficient for classifying an individual as having clinical strangles. Thus, solely finding mucopurulent or purulent nasal discharge or lymph node abscessation or rupture even in the absence of any other of the above-described clinical changes, sufficed to consider the animal strangles positive. The horses’ clinical scores from the final sampling occasion after the outbreak had abated (19 weeks after the outbreak peak) were used as controls for comparison to clinical scoring during the peak of the outbreak for the S. equi culture-positive animals.

Statistics

Descriptive statistics, including 95% confidence intervals (CI) where appropriate, were calculated. Comparison of clinical scores in the culture-positive horses to the herd baseline after recovery was performed by the nondirectional Wilcoxon signed-rank test. All calculations were performed using JMP version 12, and a P < 0.05 was deemed significant.

Results

Microbiology

At the time of nasal swab sampling, 14 of 112 animals were culture positive and 53 of 112 qPCR positive
for *S. equi*. All culture-positive horses also were qPCR positive. Additionally, 95 of 112 horses were nasal culture positive for *S. zooepidemicus*.

**Clinical Scoring and Serology**

Based on clinical scoring at the time of nasal sampling, 34 of 112 horses had clinical scores of ≥4 and thereby were classified as having clinical strangles. Of these, *S. equi* was culture positive in 4 and qPCR positive in 20 horses (Table 2). However, 9 of 14 horses culture positive for *S. equi* and 26 of 53 horses qPCR positive for *S. equi* had cumulative clinical scores only ≥2 and thereby lacked clear clinical signs suggestive of acute strangles (Table 2).

Based on continued follow-up by clinical examinations over time, 39 of 112 horses developed clinical scores ≥4 suggestive of acute strangles at any occasion, with a cumulative outbreak morbidity rate of 53%. Strikingly, of those horses nasal culture positive for *S. equi*, 12 of 14 were afebrile at the time of sampling, and 6 failed to develop clinical signs suggestive of acute strangles (Table 2).

During the appearance of the index cases 6 weeks before nasal sampling in relation to laboratory findings during an outbreak of strangles in 112 weanlings. A score of 4 or greater was used as the cutoff for clinical score suggestive of acute strangles.

<table>
<thead>
<tr>
<th>Clinical Score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>≥5</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>At nasal sampling</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All weanlings</td>
<td>5</td>
<td>26</td>
<td>31</td>
<td>16</td>
<td>4</td>
<td>30</td>
<td>112</td>
</tr>
<tr>
<td><em>S. equi</em> culture positive</td>
<td>0</td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>14/112</td>
</tr>
<tr>
<td><em>S. equi</em> qPCR positive</td>
<td>0</td>
<td>15</td>
<td>11</td>
<td>7</td>
<td>2</td>
<td>18</td>
<td>53/112</td>
</tr>
<tr>
<td><em>S. zooepidemicus</em> culture positive</td>
<td>4</td>
<td>22</td>
<td>25</td>
<td>14</td>
<td>4</td>
<td>26</td>
<td>95/112</td>
</tr>
<tr>
<td>Seropositive Ag A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>15</td>
<td>9</td>
<td>3</td>
<td>19</td>
<td>58&lt;sup&gt;a&lt;/sup&gt;/110&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Seropositive Ag C&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2&lt;sup&gt;d&lt;/sup&gt;/110&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
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</table>

Any single sample seropositive over entire 19 weeks

Ag A | 5 | 23 | 20 | 14 | 4 | 26 | 91/112 |
Ag C | 3 | 1 | 0 | 1 | 2 | 0 | 7<sup>c</sup>/112 |

<sup>a</sup>Includes 5 that were seropositive 6 weeks before nasal sampling and 31 seropositive 3 weeks before nasal sampling.

<sup>b</sup>2 samples missing analysis.

<sup>c</sup>Also seroconverted to Ag A.

<sup>d</sup>Classified as only suspicious ELISA: Optical Density <0.5 but ≥0.3

<sup>e</sup>Antigen A of the optimized ELISA to *S. equi*, tAntigen C of the optimized ELISA to *S. equi*.

During the appearance of the index cases 6 weeks before nasal sampling (Table S1, Fig 1), serology against antigens A and C of the optimized ELISA<sup>e</sup> was negative in all but 9 animals, 4 of which were classified as suspicious and 2 of the 5 fully positive with clinical strangles.

By the time of nasal sampling for *S. equi* in mid-November, >50% (58/110; 2 samples missing analysis) of the group, including 32 of 52 qPCR positive (1 qPCR positive missing serum analysis) developed seropositivity to antigen A, suggesting that the outbreak had peaked.

Once the outbreak had fully abated, 19 weeks after nasal sampling, 91 of the 112 horses had become seropositive to antigen A on at least 1 sampling occasion, with seroconversion in 51 of 53 of those that were qPCR positive at nasal sampling. All but 1 of the nasal culture-positive horses (13 of 14) eventually seroconverted to either antigen A or C. Strikingly, however, seroconversion to antigen C was minimal, with only 2 horses at the time of nasal sampling classified as “suspicious” and which were both culture and qPCR negative. In total, only 7 horses were identified as fully positive to antigen C over the entire monitoring period, and only in combination with seropositivity to antigen A (Table 2). Of these, only 2 of 7 animals attained a clinical score ≥4 at any single examination over the entire period.

Thus, in contrast to expectations from the literature, clinical signs throughout the outbreak were mild and failed to occur in almost half (6 of 14) of *S. equi* culture-positive animals at any time throughout the outbreak. Moreover, this outbreak was associated with restricted seroreactivity to the recently developed optimized *S. equi* ELISA<sup>f</sup>, which can hamper the effectiveness of control programs for management of strangles outbreaks.

**Discussion**

At the outset of this strangles outbreak, it was anticipated that the majority of weanlings would be severely affected clinically, and because of the risk of severe complications, including mortality, the group was...
monitored frequently by the veterinary staff. Thus, it was surprising that the morbidity throughout the entire outbreak was low with only mild clinical signs in affected animals, as supported by the results of clinical scoring that set a low threshold to identify suspect strangles-affected individuals. Over the course of the outbreak, several clinicians conducted the clinical examinations used for scoring. Although interobserver differences may have influenced some of the longitudinal scoring for the lymph node swelling and character of the nasal discharge, rectal temperature, white blood cell count, and presence of lymph node abscessation would be unaffected by interobserver differences. Importantly, all scoring was performed blinded to culture and qPCR results for S. equi. Because rectal temperatures were taken daily in horses with transient fever may not have been detected. However, it was still striking that 42 of 53 horses that were qPCR positive for S. equi had normal rectal temperatures on the days measurements were taken and the remaining 11 horses averaged rectal temperatures of 38°C. Thus, additional possible criteria for the onset of morbidity, a striking feature in this outbreak was that almost half (6 of 14) S. equi nasal culture-positive weanlings failed to develop clinical strangles at any time during the outbreak. Given that nasal swabs are inferior to nasopharyngeal samples for diagnosis, it is likely that there were more foals in the group that would have been S. equi nasal culture positive but were falsely negative on our sampling.

Based on current literature, introduction of S. equi to a naïve population of young horses would be anticipated to be associated with morbidity approaching 90% and even with some mortality, neither of which pated to be associated with morbidity approaching an a€escapes of immune detection accompanied by less severe clinical signs. The potential role of the SeM protein, which appeared to be associated with virulence was reported in association with a truncated isolate involved had decreased virulence of this isolate and failure to seroconvert to ELISA antibody levels in corresponded with reductions in lymph node abnormalities appearing days later. However, in such early stages of strangles, little to no bacterial shedding can be detected. Thus, because many of the horses in this outbreak were indeed shedding bacteria, sufficient time in the pathogenesis of strangles should have passed to result in the development of clinical signs. Also, because all animals except for 9 were initially serologically negative to S. equi, infections should have produced clinical signs such as fever and lymph node swelling that would persist at least several weeks, which would have been identified on clinical examinations of the group after nasal sampling. Based on longitudinal clinical monitoring and the fact that >50% of the cohort had begun to seroconvert to S. equi at the time of nasal swab sampling, the group was sampled at the peak of the outbreak.

Having eliminated the presence of pre-existing immunity and sampling times in this low morbidity outbreak, the involvement of a less pathogenic isolate of S. equi was considered. Earlier, a decrease in virulence was reported in association with a truncated SeM protein, which appeared to be associated with escape of immune detection accompanied by less severe clinical signs of strangles. In this outbreak, morbidity was only 59 of 112 (53%) despite lack of biosecurity measures to prevent spread. Moreover, in contrast to studies in other outbreaks, more half of the foals testing positive for S. equi at the peak of outbreak lacked clear clinical signs of strangles. Several possible explanations for the observed discordance between clinical severity were considered and included potential immunological protection by residual passive immunity or earlier exposure to S. equi, whether the clinical observations were too early or late in the disease process and that the S. equi isolate involved had decreased pathogenic potential. Earlier work1 suggests that protection against S. equi in young foals is not only provided by maternal immunoglobulins received during colostrum intake postnatally but also by ingestion of immunoglobulins in milk that directly coat the upper respiratory and oral mucosa. Because this group was only recently weaned, it was possible that the high levels of colostrum and milk intake, in particular, those against SeM antigens, may decrease the severity of clinical signs in foals.2,11 However, given that all but 9 of the horses in this cohort were completely seronegative to both antigens A and C of S. equi shortly before to the outbreak (Table 2), residual immunity did not likely play a role in decreasing clinical severity. Alternatively, infection by S. equi in foals before weaning could have influenced disease severity during this outbreak. However, because there is regular detailed monitoring and recording of the health of the foals until the time of weaning on this farm, earlier strangles infections would have been detected.

Because detailed clinical examination results described coincided with nasal swab sampling, the stage of disease in individuals and duration of disease could have influenced our findings. For example, in our group, clinical signs such as fever are present with nasal discharge and lymph node abnormalities appearing days later. However, in such early stages of strangles, little to no bacterial shedding can be detected. Thus, because many of the horses in this outbreak were indeed shedding bacteria, sufficient time in the pathogenesis of strangles should have passed to result in the development of clinical signs. Also, because all animals except for 9 were initially serologically negative to S. equi, infections should have produced clinical signs such as fever and lymph node swelling that would persist at least several weeks, which would have been identified on clinical examinations of the group after nasal sampling. Based on longitudinal clinical monitoring and the fact that >50% of the cohort had begun to seroconvert to S. equi at the time of nasal swab sampling, the group was sampled at the peak of the outbreak.

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dose in experimental infections necessary to induce clinical strangles.\textsuperscript{13} Although it is not feasible to determine the infective dose in natural outbreaks such as ours, other studies report the development of disease as a consequence of comingling, as occurred in this outbreak.\textsuperscript{2,14,15} With morbidity exceeding 80\%, which is considerably higher than the 53\% observed here.

Recovery of \textit{S. zooepidemicus} from nasal swabs was proportionally very high, even from animals with respiratory signs.\textsuperscript{13} It has been shown that clonal strains of SzP gene types of \textit{S. zooepidemicus} on their own can cause mild strangles-like outbreaks.\textsuperscript{17} However, preliminary inspection of the SzP from a subgroup of clinically affected versus clinically normal horse did not incriminate a particular clone (data not shown). Moreover, because most animals did eventually seroconvert to \textit{S. equi}, it is even less likely that coinfection by \textit{S. zooepidemicus} contributed to clinical disease.

Curiously, seroconversion using the optimized ELISA against \textit{S. equi} was almost exclusively restricted to antigen A (a protein fragment encoded by the gene SEQ_2190) and only occurring in combination with seropositivity to antigen A. Lack of serological response to antigen C has both biological and diagnostic implications. Earlier workers\textsuperscript{2} report that foals of similar age that develop specific IgG against the SeM protein would be protected against clinical strangles. Thus, it was initially suspected that the isolate involved in our outbreak may have undergone a genetic deletion event\textsuperscript{18} that decreased its virulence and thereby immunogenicity against the SeM protein. However, as noted above, sequencing studies of the isolate identified no genetic changes, such as deletions or point mutations, in SeM or its promoter region. Nonetheless, it is possible that there were transcriptional differences in these strains that decreased transcription of SeM leading to a decreased antibody response (personal communication, Karen Steward). Therefore, it is unclear what level of immunity the horses that recovered from strangles outbreak may have, if challenged by \textit{S. equi} infection in the future. Additionally, because the diagnostic sensitivity of the recently developed optimized ELISA relies on the additive effect of using 2 different antigens to capture all \textit{S. equi} exposed horses, loss of seroreactivity to 1 of the antigens potentially could decrease the sensitivity of this serodiagnostic testing. Although it remains to be proven, there may have been a biological association between the lack of severity of clinical disease and failure to seroconvert against the SeM protein in the strangles outbreak described here.

Conclusions

In our study, immunologically naïve foals naturally exposed to an apparently fully virulent \textit{S. equi} failed to exhibit classical clinical signs of strangles. This lack of clinical signs was also associated with incomplete serological reactivity to an enhanced serologic test for exposure to \textit{S. equi}. The important aspect in relation to disease containment and control during outbreaks is that clinical signs alone may be insufficient to release horses from quarantine measures if they have clear exposure risks to known infected horses, and in particular with the well-recognized risk of persistence as clinically silent carriers. Because most horses in the outbreak described here eventually seroconverted to 1 of the antigens in the enhanced ELISA, serodiagnosis appears to be of value in detecting exposure to \textit{S. equi} even in the absence of clinical signs. It remains to be determined whether or not a lack of antibody production against the SeM protein, as suggested by failure to seroconvert to antigen C of the enhanced ELISA, may be of consequence in the longer term protective immunity gained by horses in the outbreak. The role of the gene SEQ_0402 in the virulence of \textit{S. equi} warrants further investigation.

Footnotes

\textsuperscript{a} Swab, Amies-Coal; Sarstedt AG&Co 51588 Nürnberg, Germany
\textsuperscript{b} Sysmex KX-21N; Sysmex Deutschland GmbH, Norderstedt, Germany
\textsuperscript{c} JMP: A Business Unit of SAS SAS Campus Drive, Cary, NC 27513
\textsuperscript{d} Intervacc, Västertorpsvägen 135, SE-129 44 Hägersten

Acknowledgments

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Conflict of Interest Declaration: Authors declare no conflict of interest.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

References

6. Robinson C, Steward KF, Potts N, et al. Combining two serological assays optimises sensitivity and specificity for the


Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Clinical and ELISA findings in 14/112 weanlings nasal swab culture positive to S. equi during the peak of a strangles outbreak.

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