Papers

Use of a novel serological test for exposure to *Streptococcus equi* subspecies *equi* in hospitalised horses

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Thirty horses with no external signs of strangles were tested for exposure to *Streptococcus equi* subspecies *equi* (*S equi*) using a new, commercially available serological test. The horses were also tested for persistent carriage of *S equi* by endoscopy of the guttural pouches and PCR analysis of lavage samples. The owners were questioned about the recent medical history of the horses. Serology suggested that four horses had been recently exposed to *S equi*. None of the horses had a known history of strangles but three of the four seropositive horses had recently shown non-specific signs of respiratory disease. One asymptomatic horse was positive for *S equi* by PCR, but none had both guttural pouch abnormalities and a positive PCR result. Ten additional horses known to have strangles were all seropositive by the serological test.

STRANGLES results from infection with *Streptococcus equi* subspecies *equi* (*S equi*) and classically involves a suppurative upper respiratory tract infection associated with pyrexia, lymphadenopathy, depression and inappetence. Most horses recover from the infection over a period of two to three weeks and develop a solid immunity (Sweeney and others 2005). Following an outbreak of strangles, up to 31 per cent of horses may become carriers, in which the infection persists despite the cessation of clinical signs. Asymptomatic carriage most commonly occurs as guttural pouch empyema and has been reported to persist for up to 56 months after the initial infection (Newton and others 1997, 2000, Sweeney and others 2005).

The detection of asymptomatic carriers is difficult, as haematological and biochemical screening methods are poorly sensitive and non-specific. Bacteria may be shed intermittently, and the sensitivity of bacteriological culture of a nasopharyngeal swab is only approximately 45 per cent (Newton and others 1997). Endoscopic assessment of both guttural pouches by culture and PCR analysis of lavage samples is significantly more sensitive (Newton and others 2000). The current codes of practice on equine diseases published by the Horserace Betting Levy Board (HBLB) (2008) state that 'no convalescent horse or in-contact can be considered free from infection until three negative nasopharyngeal swabs have been obtained and the horse has been tested negative by guttural pouch endoscopic examination and lavage'. These techniques are costly, and impractical for routine screening of large numbers of horses at low risk of being infected with *S equi*.

Strangles is not a notifiable disease in the UK, and no accurate estimates exist of the prevalence of the disease or asymptomatic carriage of *S equi* in the general equine population. However, data from the Defra/Animal Health Trust (AHT)/British Equine Veterinary Association (BEVA) Equine Quarterly Disease Surveillance Reports (Defra/AHT/BEVA 2010) show that *S equi* was cultured from 805 of the 10,042 samples submitted to diagnostic laboratories throughout the UK in 2008. However, in many outbreaks, horses may suffer 'atypical' strangles, a milder or even subclinical form of disease, and *S equi* infection may not be recognised in such cases unless appropriate samples are taken (Slater 2007).

Recently a new serological test, developed by the AHT, has become available in the UK. This ELISA detects IgG antibodies to two novel *S equi*-specific antigens, and reveals recent exposure to *S equi* with a reported sensitivity of 91.5 per cent and specificity of 90.5 per cent. The test is offered as a means to screen asymptomatic horses before they are introduced to a disease-free population, such as mares travelling to a stud. Seropositive horses may pose no risk to other horses, but isolation as a precaution and further investigation by endoscopy are advised. The test primarily detects recent exposure to *S equi* but is also claimed to detect asymptomatic carriers with a sensitivity of 90.9 per cent and a specificity of 82.6 per cent (AHT 2008).

The authors' hospital is located in an area where a moderate prevalence of strangles is reported anecdotally. As part of a programme to identify and treat strangles carriers and reduce the risk of infection in the hospital, this study aimed to assess the level of seropositivity to *S equi* in a population of asymptomatic horses admitted to the hospital, and to correlate this with carrier status and the owners' awareness of exposure of their horses to strangles within the past six months.

Materials and methods

Horses with no signs of strangles that underwent elective general anaesthesia or were euthanased (for reasons unrelated to respiratory disease) by intravenous injection of secobarbital sodium and

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cinchocaine hydrochloride (Somulose; Arnolds) at Bell Equine Veterinary Clinic between July and December 2007 were eligible for inclusion in the study. Thirty horses were selected randomly from this population. A serum sample was taken from each horse for reasons unconnected with the study, and excess serum was used to quantify antibodies to *S equi* by ELISA. The test provides a result measured as the optical densities (ODs) to two *S equi*-specific antigens (A and B). For antigen A, titres above 0.5 were considered positive and titres over 1.0 were considered strongly positive; for antigen B, titres over 1.0 were considered positive and titres greater than 1.5 were considered strongly positive (A. S. Waller, unpublished data). As a positive control group, sera from 10 horses hospitalised because of *S equi* infection over the same period were analysed using the test. All positive control horses showed clinical signs of strangles and were positive for *S equi* by culture and/or PCR analysis at the time of serum collection.

Each horse was examined endoscopically either immediately after euthanasia or while under general anaesthesia. A clean, sterilised fibreoptic endoscope was introduced into each nasal cavity and the sinus outflow tracts were examined before examination of each guttural pouch. Any abnormalities were recorded, and guttural pouch lavage was performed as described by Fintl (2000). The lavage samples were tested for *S equi* DNA by PCR analysis (Animal Health Trust, Newmarket) as described by Newton and others (2000).

For the purposes of this study, horses were defined as chronic carriers if they had both endoscopically visible signs of pathology and were PCR-positive. Both criteria were required as reported cases suggest that endoscopically visible signs of pathology are very likely to be present in chronic carriers (Newton and others 1997, 2000, Fintl and others 2000) but may also result from other causes (Edwards and Greet 2007). While the authors consider the presence of *S equi* in the respiratory tract of asymptomatic horses to be abnormal, this alone is insufficient evidence of chronic carriage. A positive PCR result for *S equi* would also be expected in horses with prodromal disease.

All of the horses' owners or agents completed a questionnaire and gave their consent for inclusion in the study. The questionnaire gathered information on the horse's management and any history of respiratory disease in the horse or other horses on the same premises in the previous six months. Respiratory disease was classified as infectious, allergic or non-specific. Cases of infectious or allergic disease had to have been examined by a veterinary surgeon and diagnosed as such; in cases of non-specific respiratory disease, either the horse had not been examined by a veterinary surgeon or no diagnosis had been reached. The results of serology were not known when the questionnaire was completed. Owners of ELISA-positive horses were subsequently asked about any history of vaccination against strangles.

Results

Results of serology, endoscopy and guttural pouch lavage, and questionnaire data were available for all 30 asymptomatic horses included in the study. The horses ranged in age from eight months to 39 years, with a median of 10 years (interquartile range four to 14 years). There were 12 thoroughbreds or thoroughbred crosses, seven warmbloods, four cobs or cob crosses and seven horses or ponies of other breeds.

Serology

Four horses (13 per cent) tested positive by ELISA for one or both *S equi* antigens. All four were positive for antibody to antigen A and one of these horses was also positive to antigen B; no horses were positive to antigen B alone. The OD values for antigen A ranged from 0.560 to 0.749 with a mean of 0.650. The single positive result to antigen B had an OD of 1.612. The other 26 horses were negative for antibody to both antigens; seronegative horses had a mean OD of 0.185 to antigen A and 0.307 to antigen B.

All 10 positive control horses had seroconverted to antigen A, with a mean OD of 2.28; seven were classed as strong positives. Seven had seroconverted to antigen B (mean OD 1.96), of which six were strong positives.

Endoscopy and PCR

No horses were identified with both positive PCR results and guttural pouch abnormalities. In 20 of the horses (67 per cent), no guttural pouch abnormalities were detected and PCR was negative. In nine horses (30 per cent), guttural pouch abnormalities were detected, but PCR was negative. The guttural pouch abnormalities observed included a mild increase in mucus in one or both pouches (five horses), lymphoid hyperplasia in one or both pouches (three horses) and lymphoid hyperplasia with increased mucus in both pouches (one horse). In all but one case these abnormalities were considered mild, and no specific treatment was given. The most marked changes were in a horse with dysphagia due to a mandibular fracture and osteomyelitis. All the horses with guttural pouch abnormalities tested negative by ELISA.

One 18-month-old horse tested positive for *S equi* by PCR, but showed no guttural pouch abnormalities and had not seroconverted. This horse had been presented for a closed castration, and the PCR result was received after it was discharged from the hospital. The agent was contacted, and the horse was isolated and monitored closely for 21 days. Follow-up telephone interviews over three months confirmed that no signs of strangles had developed in this horse or among in-contact horses. For practical reasons repeat endoscopy and serology could not be performed.

Questionnaire results

There was no history of strangles in any of the asymptomatic horses or in other horses on the same premises in the six months before examination. The owner of one horse mentioned strangles outbreaks on adjoining yards; another horse had suffered from strangles 12 years previously. Both horses were negative on *S equi* serology and guttural pouch endoscopy.

Seventeen horses were kept on livery yards, 10 were kept at the owners' homes, two were on racing yards and one was at stud. The owners were asked for any history of respiratory disease in their horse in the previous six months; 22 horses (74 per cent) had shown no signs, three (10 per cent) had been diagnosed with allergic lower airway disease, one (3 per cent) had been diagnosed with a viral respiratory infection and four horses (13 per cent) had shown non-specific signs of respiratory disease.

The number of horses kept on each premises ranged from one to 85, with a median of 12. The owners were asked whether other horses on the same premises had shown signs of respiratory disease in the previous six months: 16 properties had no affected horses, allergic respiratory disease had been diagnosed on six yards, infectious respiratory disease had been diagnosed on five yards and there were non-specific or undiagnosed signs of respiratory disease on four yards. No ELISA-positive horses had been vaccinated against strangles and all had been in the owners' possession before a vaccine became temporarily available in the UK (from November 2004 to December 2006).

Analysis

The power of statistical analysis is limited by the small sample size, but these data provide a useful basis for more extensive studies. Of the four asymptomatic horses that tested positive by ELISA, three had a history of respiratory disease other than allergic disease ('non-allergic' respiratory disease) in the previous six months, compared with just one of the 26 horses that were ELISA-negative. However, there was no history of non-allergic respiratory disease among the horses that were in contact with ELISA-positive horses. Among the ELISA-negative horses there was a history of non-allergic respiratory disease in other horses on the same premises in eight cases (31 per cent). Using the definition of a carrier given above, the test had a specificity of 87 per cent for carrier detection but the sensitivity could not be calculated from these data. Among the positive control group of 10 clinically affected horses, all were positive by ELISA, giving a sensitivity of 100 per cent.

Discussion

The seroconversion of four of 26 (13 per cent) of the asymptomatic horses sampled in this study is consistent with the anecdotal reports

of a moderate prevalence of strangles in the area served by the authors' clinic. The sample population was considered representative of the overall hospital population, and may differ from the general equine population. The seroprevalence of strangles has not been reported for other asymptomatic populations of horses.

The results of this study are useful when considering the risk of nosocomial *S equi* infection in this hospital population. Endoscopy and PCR failed to identify any chronic carriers among the asymptomatic horses in the present study, or among eight other horses used in a pilot study (E. J. Knowles, unpublished data). The single PCR-positive horse raises a number of interesting questions, and highlights the need to remain alert to the possibility of *S equi* infection among hospitalised horses. Interpretation of this case is difficult. The authors speculate that the horse was incubating the disease on admission to the hospital but did not develop clinical signs, possibly due to perioperative antibiotic treatment. Alternatively, the positive result may be attributable to sample contamination or to a false-positive PCR result. This horse could have been considered a carrier of *S equi* under an alternative definition, but this would have had little effect on the parameters calculated in this study or the conclusions. In unusual cases of chronic carriage of *S equi*, the guttural pouches may appear normal but are culture-positive (Newton and others 1997). Five of six chronic carriers described by Newton and others (1997), 13 of 14 carriers described by Newton and others (2000) and all four carriers described by Fintl and others (2000) had endoscopically visible guttural pouch abnormalities.

The seroconversion of four horses in the present study, whose owners were unaware of recent exposure to strangles, is interesting. Possible reasons include exposure to the disease without the owners' knowledge, persistently elevated antibody titres from an earlier exposure to *S equi*, cross-reactivity of antibodies to other bacteria, and the owners answering the questionnaire dishonestly.

Exposure of a horse to strangles without the knowledge of the owner may occur commonly. This seems particularly likely, given the high prevalence of non-allergic respiratory disease reported among the horses that had seroconverted. Many outbreaks in the UK are thought to involve atypical strangles (Slater 2007), and there have been anecdotal reports of an increase in the prevalence of atypical strangles compared with the more overt 'classical' form of the disease. However, there were no reports of non-allergic respiratory disease among other horses on the same premises.

Previously, variations between strains of *S equi*, particularly differences in the hyaluronic acid content of the bacterial capsule, were considered to be responsible for atypical strangles (Prescott and others 1982, Grant and others 1993). However, within an outbreak, the severity of disease may vary widely between horses, and host factors appear to be highly significant (Newton and others 1997, 2000, Fintl and others 2000). Compared with *Streptococcus equi* subspecies *zooepidemicus* (*S zooepidemicus*), *S equi* shows very little strain diversity (Galan and Timoney 1988), although sequence analysis of the *SeM* gene has identified at least 66 strains of *S equi* from cases of natural infection (Kelly and others 2006, Anon 2008). To date, strain variations have not been linked with different disease presentations. The most significant host factor in natural outbreaks of strangles is likely to be pre-existing immunity, the levels of which have not been reported in the general equine population. The mechanism of resistance is not fully understood and may not correlate with serum levels of IgG (Galan and Timoney 1985, Timoney and others 2007). Atypical strangles thus remains poorly understood, and warrants further investigation.

The positive ELISA results may have been due to persistently raised levels of antibody from an earlier exposure to *S equi*. The time after which antibody levels wane varies between horses, and multiple exposures may cause seroconversion for prolonged periods (A. S. Waller, unpublished data). False-positive results due to cross-reactivity of antibodies to the antigens used in the ELISA seem unlikely. The antigens have been identified as *S equi*-specific by sequence analysis and population screening of 142 diverse strains of *S zooepidemicus* (A. S. Waller, unpublished data), although the possibility of cross-reactivity cannot be completely excluded. *S zooepidemicus* is associated with non-specific equine respiratory disease (Wood and others 1993) and contains a near-

identical C-terminal portion of the SeM protein, known as SzM (Kelly and others 2006). Serological tests for *S equi* based on SeM protein may therefore be affected by cross-reactivity to SzM (Waller and Jolley 2007). The AHT's serological test is not based on SeM for this reason.

The estimate of specificity for detection of the carrier state provided by these data (87 per cent) is consistent with that reported by the test providers (82.6 per cent). The test sensitivity could not be calculated for the asymptomatic study population. The 100 per cent specificity calculated for the positive control group is of limited value. The positive predictive value (PPV) and negative predictive value (NPV) may be more valuable parameters than the sensitivity and specificity when considering a test's clinical value (Begg 1987), and these parameters vary according to the prevalence of the condition in the population being tested. A PPV of 0.68 and NPV of 0.96 for carrier detection were obtained by the providers of the test (AHT 2008). Among the 1611 samples used to validate the test, one-third were from horses known to have been exposed to strangles recently (A. S. Waller, unpublished data). The prevalence of the carrier status (and thus the test's predictive values for detecting carriers) among these horses is likely to differ from that in other equine populations. The lack of chronic carriers detected in the present study suggests a low prevalence of carriers in the population of horses, and therefore a low PPV for the test. If this test were used in a population with a carrier prevalence of 5 per cent, then 78 per cent of positive test results would not be carriers (PPV 0.22, NPV 0.99). Importantly, this test is primarily offered to screen for exposure to strangles, rather than to test specifically for carriers. Endoscopy and guttural pouch lavage remain the 'gold standard' techniques for detecting strangles carriers (Newton and others 2000, Davidson and others 2008) and this serological test should aid in the selection of cases to examine endoscopically. Ultimately, it is hoped that antibody isotyping will allow the detection of a 'carrier-specific' immune response, rather than, as with the current test, exposure to *S equi*.

The relatively high prevalence of guttural pouch abnormalities, which were observed in nine of the horses (30 per cent), is interesting, although eight of these horses showed only mild changes. There was also no association between guttural pouch abnormalities and previous disease either in that horse or among in-contact horses. All the horses with guttural pouch abnormalities tested negative by ELISA. The horse with the most prominent guttural pouch abnormalities had localised lymphadenopathy secondary to mandibular osteomyelitis and increased mucus due to a decreased frequency of swallowing. Seven of the remaining eight horses had shown no signs of respiratory disease, and their guttural pouch changes may be attributable to subclinical infectious disease or reduced mucus clearance. The authors speculate that mucus clearance may be reduced in horses that carry their heads abnormally high or that have a reduced swallowing frequency due to a reduced frequency with which the guttural pouch ostia open. Mucosal inflammation in the guttural pouch may be a feature of most bacterial and viral infections of the upper respiratory tract (Edwards and Greet 2007).

It is possible that the methods employed in the present study were inadequately sensitive. The possibility of carriage of *S equi* at a site other than the guttural pouches cannot be excluded but is unlikely. The sinus outflow tracts of all the horses were examined and showed no evidence of increased discharge. Newton and others (1997) reported the carriage of *S equi* in one pony in which the bacterium was not isolated from the guttural pouch; however, in that case guttural pouch endoscopy was performed only after antibiotic treatment had begun. PCR analysis of guttural pouch lavage samples is considered to be highly sensitive (>90 per cent) for both live and dead bacteria (Newton and others 2000; A. S. Waller, unpublished data).

In summary, the asymptomatic horses that were positive by ELISA had a high prevalence of undiagnosed respiratory disease in the previous six months (three of four horses). The apparent presence of exposure to *S equi* exposure without the owners' awareness, and the detection of *S equi* DNA in one seronegative horse, highlight the need to remain alert to the possibility of strangles infection among hospitalised horses. In this study, no positive ELISA results were associated with subclinical guttural pouch empyema. Positive ELISA results should be interpreted with caution as an indication for the isolation of horses and endoscopy and further diagnostic procedures, but not specifically of carrier status.

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