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Repeated nasopharyngeal lavage predicts freedom from silent carriage of *Streptococcus equi* after a strangles outbreak

John Pringle[1] | Anna Aspán[2] | Miia Riihimäki[1]

[1]Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences, Uppsala, Sweden

[2]The National Veterinary Institute, Uppsala, Sweden

Correspondence
John Pringle, Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences, Box 7054, 750 07 Uppsala, Sweden.
Email: john.pringle@slu.se

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Abstract

Background: The value of repeated nasopharyngeal lavage (NPL) to detect silent carriers of *Streptococcus equi* has not been investigated.

Hypothesis/Objectives: Determine if results of serial testing for *S. equi* by NPL predicts subsequent true carrier status as determined by both NPL and guttural pouch lavage.

Animals: An outbreak of strangles with 100% morbidity in 41 mature Icelandic horses was followed prospectively to investigate development of silent carriers. All were initially positive to *S. equi* on NPL. The farm was closed to horse movement during the entire study.

Methods: Prospective observational study. Testing for *S. equi* was performed by NPL at weeks 18, 28, 29, and 30 postindex case and subsequently at week 45 by both NPL and guttural pouch lavage. Carrier status at week 45 was compared to results obtained at weeks 18, 28, 29, and 30. Descriptive statistics were calculated. Comparisons were made using Fisher's exact test or the Freeman-Halton extension with a $P < .05$ level of significance.

Results: Of 24 noncarriers at week 45, only 4 horses were negative on all 3 consecutive weekly NPL samples at weeks 28 to 30. However, 10 of the 11 horses with at least 3 negative NPL obtained from weeks 18, 28, 29, and 30 were *S. equi*-free at week 45 ($P = .03$).

Conclusions and Clinical Importance: Repeated NPL on at least 3 separate occasions can assist in predicting *S. equi* carrier-free status in horses after recovery from a strangles outbreak.

KEYWORDS
clinical trials, evidence based medicine, nasopharyngeal lavage, silent carrier, strangles

1 | INTRODUCTION

Strangles is a highly infectious upper respiratory tract disease in horses throughout most of the world, caused by the β-hemolytic Lancefield group C bacteria, *Streptococcus equi* subspecies *equi* (hereafter *S. equi*). Acutely, the disease is associated with fever, lethargy and swollen abscessed lymph nodes over a period of weeks or even months[1] after which most horses recover uneventfully. Recognition of the carrier state[2] (predicted early in the 1900's[3]) and awareness that the organism could persist in the guttural pouches[4] were key advances in understanding the overall epizootiology of strangles and how it may persist and spread to immunologically

Abbreviations: GPL, guttural pouch lavage; NPL, nasopharyngeal lavage.

susceptible horses. Carriers are undoubtedly key offenders in the spread of strangles to naïve horses, and indeed persistence of this disease worldwide.[5]

Two recent American College of Veterinary Internal Medicine (ACVIM)consensus statements on strangles management indicate that detection of the carrier state for *S. equi* requires either repeated nasopharyngeal lavage (NPL) or direct sampling from the guttural pouch. The initial consensus statement of 2005[6] advised that consistent negatives for *S. equi* on PCR from 3 consecutive weekly sampling of nasopharyngeal swabs or lavages were sufficient to allow horses to return to a "clean" noninfected group. On the other hand, the more recent consensus statement[7] suggested that qPCR on a single endoscopically-guided guttural pouch lavage (GPL) would provide increased efficiency and sensitivity over 3 NPLs over 3 weeks to screen for carriers. Unfortunately, neither of these publications provided evidence based studies to support the statements. Although clearly important in carrier detection, GPL is more invasive and costly. Also, use of an endoscope can result in false positives.[8] Nonetheless, although single sampling by NPL risks incurring false negatives,[9] the method is relatively simple, inexpensive, minimally invasive,[10] and readily repeated over time. Moreover, it has been shown that combined sampling by both NPL and GPL is required for optimal testing for the carrier state because single guttural pouch samples also can be falsely negative in silent carriers of *S. equi*.[9] Other minimally invasive alternatives to carrier testing, such as use of serology after strangles outbreaks, have lacked clinically useful discriminatory power because carriers can be seronegative,[11] even when culture positive.[12]

Thus, there is a pressing need to critically examine simpler methods of poststrangles testing after natural outbreaks in relation to their prediction of carrier-free status.

We conducted a prospective longitudinal study of development of the carrier state in a strangles outbreak with 100% morbidity in a closed group of 41 mature Icelandic riding horses. The clinical progress of the horses was followed from the index case to full recovery. Carrier status for each horse was determined by bacterial culture and qPCR of 4 sequential NPLs and finally by both NPL and guttural pouch endoscopy and lavage. We examined whether results of serial sampling for *S. equi* by NPL after clinical recovery in this herd could predict carrier-free status, as determined by an optimal combination of sampling for carriers that includes both NPL and GPL.[9]

2 | MATERIAL AND METHODS

2.1 | Horses included in the study

A strangles outbreak with 100% morbidity occurred in a closed group of 43 adult Icelandic horses. Two horses were euthanized at the onset of the outbreak for economic and ethical reasons. The remaining 41 horses (mean, SD age 16.0, 6.6 years; median, 14; range, 7-32 years), including 32 geldings and 9 mares, were followed prospectively from acute illness until clinical recovery by upper respiratory microbiology to identify and manage potential silent carriers. All horses were *S. equi*-positive by culture or qPCR on NPL during the acute phase (first 7 weeks postindex case; Figure 1). Because all horses were clinically affected in this outbreak, clinically diseased horses were not isolated. In the stable, all horses had individual boxes with low walls that allowed nose-to-nose contact with nearby stablemates. Three horses were lost for final sampling 45 weeks after the index case (see clinical sampling below), with 2 horses euthanized; 1 because of laminitis and the other because of peritonitis. The remaining horse was moved to another farm after being found to be *S. equi*-negative at $T = 30$ (see below).

2.2 | Clinical sampling

Data included in the study are from 5 herd visits, hereafter denoted sampling occasions based on number of weeks after the initial outbreak, with $T = 0$ representing the index case. Samplings $T = 18$, 28, 29, and 30 were conducted after all clinical signs of strangles had resolved. Because of continued detection of many carriers, and few horses consistently free of *S. equi* on NPL (see Supporting information), the farm remained closed to movement over the winter season to allow additional time for carrier convalescence before retesting all horses at $T = 45$. At that time, the optimal combination of tests, including both NPL and GPL, was used to reliably differentiate carriers from *S. equi*-free animals (Figure 1). On each sampling occasion all horses had complete clinical examinations performed with clinical scoring assigned as previously described.[13] Nasopharyngeal lavages were performed on all horses on all sampling occasions whereas at $T = 45$ GPLs for *S. equi* also were conducted on all horses remaining on the premises. During the entire study, personnel used disposable protective clothing and changed gloves between horses. Nasopharyngeal lavage was performed as described elsewhere[10] by instilling 250 mL NaCl (0.9%) via a new single use foal feeding tube (Vycom REF 310.12) at the level of the nasopharynx with fluid recovered collected in a disposable plastic bag held over the nares and transferred to sterile 50 mL plastic tubes (Sarstedts REF 547.004). Guttural pouch lavages were performed endoscopically on each guttural pouch with 40 mL sterile saline infused via disposable silastic tubing positioned within the biopsy channel, aspirated, and stored separately for analysis. Three different endoscopes were used with sterilization between use by immersion and channel flushing with orthophthalaldehyde disinfectant (Cidex OPA Johnson & Johnson AB). All lavage fluids collected were centrifuged and resulting cell pellets used for further analysis.

All samples were stored at +4°C and on the following day analyzed for *S. equi* by qPCR[14] and bacterial culture at the Swedish National Veterinary Institute, Uppsala, Sweden.

A carrier of *S. equi* was defined as any animal found positive by culture or PCR on any test sample at $T = 45$.

2.3 | Statistics

Descriptive statistics were calculated and the results of horses found positive for *S. equi* at each sampling ($T = 18$, 28, 29, 30) compared to

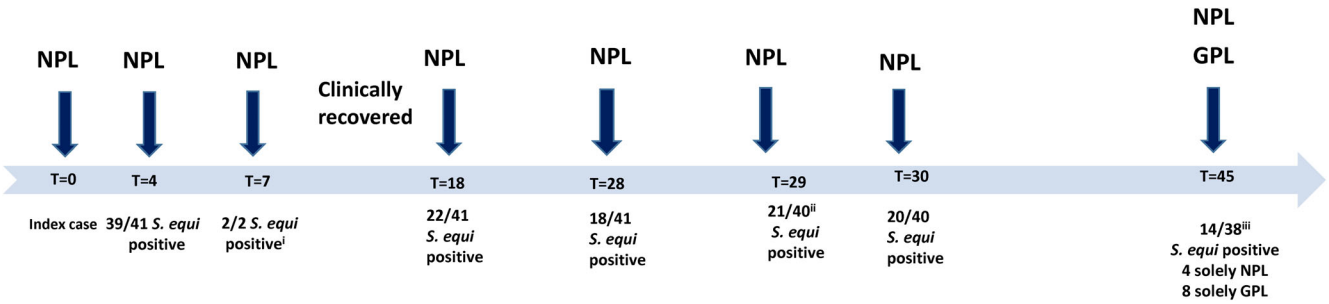

FIGURE 1 Time scale with T = weeks after index case ($T = 0$) for sampling of the strangles outbreak in 41 mature Icelandic horses, and testing for presence of *Streptococcus equi* from acute disease to full clinical recovery. [i]Resampling of the sole 2 horses PCR negative at T4, [ii]one euthanized due to laminitis, [iii]two lost to follow up sampling; one moved off farm, the other euthanized. GPL, guttural pouch lavage; NPL, nasopharyngeal lavage

results from those identified positive for *S. equi* at week $T = 45$. After initial analysis determined that only 4 horses were *S. equi*-negative on all sampling occasions over the 3-week interval 28 to 30 as suggested previously,[6] the results from all 4 NPL samplings at $T = 18$ to 30 were included in the analysis and compared to those horses found *S. equi*-free at $T = 45$ (noncarriers). Comparisons between groups was performed using the 2-tailed Fisher exact probability test or the Freeman-Halton extension. The level of significance was set at $P < .05$.

The study was approved by the Swedish Ethical Committee on Animal Experiments (diary number C 36/14). Horse owners provided informed consent for use of their animals in the study.

3 | RESULTS

At $T = 45$, 24 of the 38 horses remaining on the farm were found to be free of *S. equi*. Of the 14 positive results identified, 8 were PCR positive for *S. equi* solely on GPL samples, whereas 4 were positive solely on NPL (Figure 1). There were 22/41 horses *S. equi*-positive on NPL at $T = 18$, 18/41 at $T = 28$, 21/40 at $T = 29$, and 20/40 at $T = 30$. Of the 38 horses sampled 4 times ($T = 18$, 28, 29, 30) and included at $T = 45$, 4 were consistently negative, 4 were consistently positive, and the remaining 30 varied between 1 to 3 tests negative for *S. equi* (see Supporting information).

When restricted to findings of weekly sampling over the 3 continuous weeks ($T = 28$-30), 4 horses were consistently negative for *S. equi*, and in turn, all were negative at $T = 45$ (Table 1). Alternatively, of the 16 horses with 2 negative samples between $T = 28$ and 30, 11/16 were *S. equi*-negative at $T = 45$. Within the remaining 18 horses with 1 or no negative sample for *S. equi*, 9 still were *S. equi*-negative at $T = 45$. Despite the apparent numerical differences, no statistical differences were found between these groups in the proportion that were *S. equi*-negative at $T = 45$ ($P = .15$, Freeman-Haltmann extension of Fisher's exact test).

When including results from the additional NPL sampling at $T = 18$, 11 horses had at least 3 of 4 samples negative. All but 1 of these were *S. equi*-negative at $T = 45$, whereas approximately one-half of those with <3 negative samples (13/27) were positive for *S. equi* at $T = 45$ ($P = .03$; Fisher's exact test, Table 2).

TABLE 1 Freedom from *Streptococcus equi* at $T = 45$ in relation to a 3-week sequential sampling by nasopharyngeal lavage at weeks $T = 28$, 29, and 30

Number *S. equi* negative	*S. equi* negative $T = 45$	*S. equi* positive $T = 45$
3	4[a]	0
2	11[a]	5
≤1	9[a]	9

[a]$P = .15$ Freeman-Halton extension of the Fisher exact probability test.

TABLE 2 Results of NPL or GPL for *Streptococcus equi* at $T = 45$ in relation to cumulative number testing *S. equi* negative over 4 previous sampling (weeks $T = 18$, 28, 29, and 30) by nasopharyngeal lavage

Number *S. equi* negative	*S. equi* negative $T = 45$	*S. equi* positive $T = 45$
≥3	10[a]	1
≤2	14[a]	13

Abbreviations: GPL, guttural pouch lavage; NPL, nasopharyngeal lavage.
[a]Fishers exact $P = .03$.

4 | DISCUSSION

Our results provide supportive evidence that at least 3 repeated NPLs negative for *S. equi* on PCR collected between weeks 18 and 30 post-index case in horses after a strangles outbreak can predict *S. equi*-free status. Importantly, if borne out in additional prospective studies, incorporation of repeated NPL to determine carrier-free status can help with herd segregation between carriers and noncarriers of *S. equi*. Because the findings were compared against an optimal diagnostic panel for carrier detection, using the combination of NPL and GPL, it is unlikely that any of the 24 horses classified as carrier-free were falsely negative.

Although our findings were consistent with earlier suggestions[6] that freedom from *S. equi* could be based on 3 consecutive weekly

NPL *S. equi* negative samples, only a limited proportion of our noncarriers (4/24) were negative over the 3 consecutive weeks of sampling. However, when combining results from a fourth NPL, 10 of the 11 horses with at least 3 of the 4 samples collected between $T = 18$ and 30 testing negative for *S. equi* by PCR also were *S. equi* negative at $T = 45$, being substantially better at predicting carrier-free status than horses with ≤2 NPL-negative results for *S. equi*. Nonetheless, even then the majority of noncarriers (14/24) still were not identified by this approach (Table 2).

A key limitation of our study was the time delay between the sequential sampling and final panel of testing for silent carriers. During the time period $T = 30$ to 45 allotted for additional convalescence, cohabitation of carriers and noncarriers provided the potential for interanimal spread with those carrier-free becoming reinfected.[15] However, such reinfection should have contributed to a stable number or increase in the number of carriers detected at week 45. Moreover, the 4 horses found NPL negative at the 3-week serial testing, as well as 10 of the 11 horses with at least 3 negatives on 4 separate NPL, remained carrier-free (Tables 1 and 2), which suggests lack of recontamination by herdmates during this time gap. Another confounding aspect is that, during the time between $T = 18$ and $T = 45$, horses with low levels of *S. equi* in their upper respiratory tract may have recovered spontaneously and no longer carry *S. equi*. Although cross infection appeared unlikely, it remains to be determined if time delay was a key factor in decreasing the number of horses remaining positive for *S. equi* at $T = 45$.

Additionally, our findings represent a single strangles outbreak in a single horse breed and with unusually high morbidity. As such, direct application of our results to the wider horse population awaits additional similar studies. With respect to the recent ACVIM consensus statement[7] recommendation for reliance on carrier detection by single GPL, our results show that 4/14 (29%) of carriers would not have been detected at $T = 45$ based solely on GPL (Figure 1). Nasopharyngeal lavage samples a large portion of the nasal cavity, but does not provide access to the guttural pouches. As such, secretions containing *S. equi* emanating from the GP likely are shed intermittently into the region sampled by NPL. Alternatively, *S. equi* recovered in NPL but not found on GPL may originate from nonguttural pouch sites, such as the nasal sinuses. Unfortunately, the site of origin in horses that are NPL-positive but GPL-negative, remains to be determined. Although others[16] have suggested exclusive reliance on GPL for carrier detection, horses in that study were examined at a median time of 3 months from initial infection, with the majority of horses still having visual abnormalities within the guttural pouches, and as such may have overlapped with horses undergoing natural recovery from strangles. In contrast, examination for carrier state in our study was not even initiated for an additional 6 weeks, which provided considerable more time poststrangles for self-recovery. Unfortunately, as yet there is no consensus on the time frame after acute strangles that separates findings in horses with natural recovery from those that have become long-term silent carriers.

In the future, hypothesis-based studies are needed to more directly compare and contrast diagnostic yield of GPL compared to repeated NPL. Clearly, serial sampling of guttural pouches needs to be performed prospectively to better define the number of false negatives in more than just a single outbreak. However, given cost and invasiveness, along with the documented risk of false positives with endoscope contamination,[8] applying serial GPL for carriers in a clinical setting has clear practical and diagnostic limitations. Thus, given its ease of use, limited invasiveness, and repeatability, serial NPL to predict carrier-free status deserves additional study and validation. Moreover, with appropriate attention to procedures that limit *S. equi* contamination of fomites,[17] such as use of protective clothing and glove changes between sampling, there should be minimal risk of false positives that can complicate endoscopic methods.[8] In particular, application of repeated NPL would allow progressive movement of those horses with 3 negative tests to clean areas, while testing by NPL could continue within the remainder of the herd to identify additional horses that have become carrier-free .

Future studies on simplifying and refining silent carrier detection also may benefit from alternative sampling methods, such as combined irrigation of guttural pouches that in turn floods the nasal cavity before collection of a single sample,[18] representing both the guttural pouch and nasal cavity. Importantly, such sampling methodology could be performed with simple instrumentation, obviating the expense and risk of false positives associated with endoscopic methods and providing a pooled sample from both guttural pouches and the nasopharynx. Although such a method would preclude visual detection of the chondroids, such changes are reported to represent only a small proportion of horses identified as silent carriers.[12,16]

On the other hand, although use of other less invasive markers of silent carrier status, such as serology, continue to be recommended,[18] hypothesis-based studies have failed to support their clinical value in identification of carriers[11] or distinguishing noncarrier herdmates poststrangles.[12]

5 | CONCLUSION

Repeated NPL with at least 3 of 4 samples negative between weeks 18 and 30 was a promising predictor of carrier-free status at week 45. However, it was only found in 10/24 noncarriers. Moreover, given that 1 carrier also was negative in 3 prior samples, additional testing by NPL would be advisable to verify carrier-free status. On the other hand, our findings do not support sole reliance on GPL on a single occasion for optimal detection of carriers because 4/14 carriers only were detected by NPL. Prospective testing in future outbreaks is needed to confirm the validity of serial NPL and GPL in determining *S. equi* carrier-free status.

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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.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the Swedish Ethical Committee on Animal Experiments (diary nr C 36/14). Horse owners provided informed consent for use of their animals in the study.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

ORCID

John Pringle https://orcid.org/0000-0003-4013-0363

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SUPPORTING INFORMATION

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