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Long term dynamics of a *Streptococcus equi* ssp *equi* outbreak, assessed by qPCR and culture and seM sequencing in silent carriers of strangles

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ABSTRACT

The aim of the study was to use culture, qPCR and seM sequencing to map *Streptococcus equi* subspec. *equi* (*S.equi*) isolates in long term carrier animals. A strangles outbreak affecting 41 Icelandic horses was followed to determine strangles free status using nasal and/or guttural pouch lavages collected serially on eleven separate occasions over 13 months. Ten persistent carriers, of which eight had repeated culture positive samples for *S. equi,* were selected for the study. Of 115 samples collected, 61 were *S. equi* positive on qPCR; from which 32 were also culture positive. Amplification of parts of the gene encoding the M-protein seM was performed on isolated colony material (n = 32) or, where only PCR product was obtained, directly on the DNA sample (n = 29) with a nested amplification approach. The seM sequence could be determined for six of the 29 samples that were solely qPCR positive. The outbreak was due to a *S. equi* strain of seM type 72. Three months after initial sampling isolates from two horses had seM gene sequences with one amino acid change. After six months *S. equi* with truncated seM genes were found in two horses; one variant in a single horse once, and in the other horse a variant that persisted and that was later identified in two additional horses. Non- mucoid *S. equi* colonies were found in two horses. Importantly, after acute strangles outbreaks many horses not only remain persistently qPCR positive for *S. equi* but are also intermittently culture positive.

1. Introduction

Strangles is a highly infectious upper respiratory tract disease in horses caused by the β-hemolytic Lancefield group C bacteria, *Streptococcus equi* subspecies *equi* (hereafter *S. equi*). Clinical strangles is associated with considerable suffering due to fever, depression and swollen abscessed lymph nodes in a horse over a period of weeks or even months (Boyle et al., 2018; Sweeney et al., 2005) after which most horses recover uneventfully. However, some horses recovered from strangles continue to carry bacteria in their upper airways without clinical signs of disease, so called “silent carriers” (Boyle et al., 2016, 2017b; Chanter et al., 2000). Recent findings, with samples analyzed by qPCR technique in long-term follow up studies, reveal that the silent carrier state is far more common[1] than previously reported (Newton et al., 1997; Sweeney et al., 2005). Moreover, these silent carriers of *S. equi* are increasingly recognized as a key factor in spread of strangles to naïve horses and maintenance of continued strangles outbreaks.

For tracing outbreak origins typing of the surface wall associated-protein M protein (seM), supposedly a key virulence factor of *S. equi* (Boschwitz and Timoney, 1994a, b; Meehan et al., 2001) has been of particular value (Ivens et al., 2011; Libardoni et al., 2013; Lindahl et al., 2011; Moloney et al., 2013; Patty and Cursons, 2014). Whereas *S. equi* is clonal according to multilocus sequence typing (Webb et al., 2008) there are currently 135 seM alleles described in the seM database (01-06-2018) (http://pubmlst.org). Recently a cross sectional study including genome sequencing of over 200 isolates of *S. equi* based on isolates submitted to diagnostic laboratories from several different countries has been performed to elucidate the global population structure of this bacterium (Harris et al., 2015). Differences in some specific genes were apparent when comparing isolates from acute clinical disease and from silent carriers. However, longitudinal studies of *S. equi* infected horses from acute to silent carrier state with well-defined clinical phenotypes, and from a closed horse stable, remain to be performed.

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[1] Pringle et al. Optimal detection of silent carriers of *Streptococcus equi* subsp *equi* in horses following strangles outbreaks. Havemeyer Foundation “Getting to grips with Strangles and other Streptococcal Diseases”. Sept 19-21, 2017 Gallatin Gateway MN USA pp 31-32

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In chronic carriers, bacteria persist mainly in guttural pouches, and are often refractory to detection by bacterial culture and only positive on PCR (Boyle et al., 2016). However, when detected only by qPCR it is unclear whether further sampling can still yield culture positives to *S. equi*. Moreover, those bacteria recovered from carriers often differ in colony appearance when cultured, which has been suggested to be related to mutations and deletions within the *has* operons, coding for enzymes involved in the production of hyaluronic acid precursors (Chanter et al., 2000; Harris et al., 2015). However, despite recent genome studies of functional essential genes and seM typing of *S. equi* in carrier animals, further studies that characterize and identify virulence factors for *S. equi* in carrier animals through genome analysis are needed to understand infection biology behind silent carriers of strangles.

For those clinically healthy silent carriers that remain qPCR positive but culture negative for *S. equi* months after clinical disease key questions arise as to whether horses solely qPCR positive for *S. equi* were falsely culture negative and whether they pose a risk of spread of infection.[2] Given that seM sequencing on DNA extracts from clinical samples of horses with strangles may differentiate between various isolates it is feasible to map the persistence and potential spread of isolates in individual horses over the course of strangles outbreaks into silent carrier state. The aim of the study was to chart the dynamics of *S. equi* based on culture, qPCR and seM sequencing in horses involved in a strangles outbreak from their early clinically diseased state through to full clinical recovery and development into clinically silent carriers.

2. Material and methods

This study was approved by the Swedish Ethical Committee on Animal Experiments (diary nr C 36/14). Horse owners provided informed consent to participate in the study.

2.1. Horses included in the study

A clinically severe strangles outbreak in 41 Icelandic horses (mean age 16,0 ± SD 6,6), with 100% morbidity, was followed prospectively over 13 months (first sampling one month after index case), with the last positive sampling performed in June 2016, 14 months after disease outbreak (Fig. 1). All horses were confirmed as being *S. equi* positive by culture. Ten months after the index case 37% (14/38) of horses were identified as carriers with the final carrier horse eventually negative to *S. equi* on sampling 17 months (data not shown) after the index case.

Ten horses in the silent carrier group with repeated culture positive samples from either nasal and/or guttural pouch lavages for *S. equi* during a 13 month follow up period were selected for this study. The mean age of the horses was 14.5 ± SD 5.9 years, including five geldings and five mares. Individuals were assigned a clinical score as previously described (Tscheschlok et al., 2018; Waller et al., 2007) based on clinical observations recorded on each animal during each of the specific clinical examination and sampling occasions. At the end of the study period (11–13 months after the index case) individual asymptomatic carrier horses were treated with a variety of antibiotic regimens (Table 2), including benzylpenicillin procaine, 20 mg/kg im q24 h, 10 days, trimethroprim sulfadiazine 30 mg/kg po q12 h, 10 days or local topical treatment with gelatin/penicillin (Verheyen et al., 2000).

As all horses were clinically affected in this outbreak there was no isolation of clinically diseased horses and same stable personnel handled all the horses in the yard. When outdoors the horses were held in smaller gender based groups but there was possibility for sharing water buckets. In the stable, all horses had individual boxes with low walls that allowed nose-to-nose contact with horses from other paddock groups. However, at the end of the outbreak (spring 2016) silent carrier horses were held on a separate pasture fully isolated from all other horses.

2.2. Sample collection

Diagnostic samples were obtained from all horses by nasopharyngeal lavages (hereafter NL) on eleven separate occasions during a 13 months period. Sampling also included guttural pouch lavage (hereafter GPL) on all carrier horses, from the 8th sampling (approximately 10 months after index case) occasion onward until the resolution of carrier state, with the last sampling performed 14 months after initial disease outbreak (Fig. 1). The majority of GPL's were performed at the end of the study period in order to optimally identify the silent carrier animals. During the study those personnel that performed sampling used protective clothing and changed gloves between horses. Nasopharyngeal lavage was performed by instilling 250 ml NaCl (0,9%) via a foal feeding tube, (Vycom REF 310.12) at the level of the nasopharynx as per Lindahl et al., 2013 (Lindahl et al., 2013) with fluid recovery to a plastic bag and transferred to sterile 50 ml plastic tubes (Sarstedts REF 547.004).

Following NL, endoscopic examination, when applied was performed by visualization of the pharynx and guttural pouches followed by lavage of guttural pouches using 40 ml NaCl (0.9%) for each guttural pouch via a single use plastic tube (Intramedic REF 41830-1) placed through the endoscope's biopsy channel. Visual appearance of the guttural pouches was graded by experienced endoscopists blinded to carrier status and according to a previously reported scheme (Newton et al., 2000). Briefly, a grade of 0 was assigned for no abnormalities, 1 if there was any trace of cloudy mucous in either guttural pouch, and 2 for presence of moderate to marked amount of purulent exudate, at least one chondroid, or residual mucosal scarring over the region of the retropharyngeal lymph nodes. On all eleven occasions NL were obtained, and GPL was performed on all horses at least once to declare the carrier free status. At the end of the follow up period (the 8th sampling; approximately 10 months after index case) individual horses were sampled until establishment of carrier free status (negative by qPCR on both NL and GPL samples). For GPL three different endoscopes, including one video scope (Eickemeyer®, endoscope A) and two fiber optic endoscopes (Olympus®, Endoscope B and C), were used for sequential sampling to allow disinfection of each endoscope prior to use on a subsequent horse. The time point of examination during the day and which of the three endoscopes used was recorded. After each examination the endoscope was manually cleaned externally with distilled H_20 and clean paper. Mechanical cleaning was followed by flushing the working channel (60 ml) and by immersion of the insertion tube in a shallow plastic bucket containing ortho-phthalaldehyde disinfectant (CIDEX® OPA ASP, Johnson & Johnson AB) for at least 20 min. Prior to use the endoscope was removed from the bucket and residual disinfectant was flushed from the surface with liberal quantities of sterile water, followed by flushing the working biopsy channel with 60 ml of distilled H_20. New disposable gloves and protective gowns were worn for each horse sampled.

2.3. Sample analysis

All samples were stored at + 4 °C and analyzed the following day for *S. equi* by q-PCR (Baverud et al., 2007) at National Veterinary Institute, Uppsala, Sweden. Samples positive for the *SeeI* gene (Ct values < 35) were cultured to obtain isolates of *S. equi* (Baverud et al., 2007). DNA extracted from swabs / lavage samples and isolated bacterial strains were stored at -70 °C for later analysis. Amplification of parts of the gene encoding the M-protein seM was performed either on isolated colony material, or, if no colonies could be isolated, directly on

[2] Pringle et al. Optimal detection of silent carriers of *Streptococcus equi* subsp *equi* in horses following strangles outbreaks. Havemeyer Foundation "Getting to grips with Strangles and other Streptococcal Diseases". Sept 19-21, 2017 Gallatin Gateway MN USA pp 31-32