Title:

New perspectives for the diagnosis, control, treatment and prevention of Strangles in horses

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Synopsis:

Strangles, characterized by abscessation of the lymph nodes of the head and neck, is the most frequently diagnosed infectious disease of horses worldwide. All horses can be affected on some yards and mortality rates can be as high as 10%. Consequently, strangles is responsible for considerable economic and welfare cost. The persistence of the causative agent, *Streptococcus equi*, in a proportion of convalescent horses plays a critical role in the recurrence and spread of disease. Recent research has greatly increased our understanding of strangles, leading to the development of effective diagnostic tests that assist the eradication of *S. equi* from local horse populations. This article describes how these advances have been made and provides practical advice to assist the resolution and prevention of outbreaks. New perspectives on preventative vaccines and therapeutic interventions are discussed towards getting to grips with strangles.

Key Points

- The ability of *S. equi* to establish persistent infection, usually within the guttural pouches, is critical to inter-epizootic transmission, the recurrence of strangles and the high incidence of this disease around the world.
- The lack of clinical signs exhibited by persistently infected carriers emphasizes the need to implement effective quarantine and testing procedures for their identification and treatment before they come into contact with an existing herd.
- A blood sample taken on arrival can be tested to identify horses that may have been recently exposed to or are persistently infected with *S. equi*, which require further investigation.
- Quantitative PCR tests for *S. equi* are now regarded as the gold-standard for the detection of *S. equi*.
- The development of effective vaccines against strangles that permit the differentiation of infected from vaccinated animals remains a significant unmet objective.

Introduction

Strangles was first reported by Jordanus Ruffus[1], an officer in the imperial court of Emperor Frederick II, in 1251 although the disease almost certainly has older origins. Despite improvements in the health and management of horse populations, strangles remains the most frequently diagnosed infectious disease of horses world-wide. Only the geographically isolated Icelandic horse population remains free of strangles, a situation that has been maintained through a virtual absence of horse import for over 1,000 years. In excess of 600 outbreaks of strangles are estimated to occur in the UK alone each year[2]. Outbreaks can involve all of the horses on a yard, require movement restrictions that often remain in force for over 2 months and an economic cost to some premises that may exceed £250,000 ($425,000).

The causal agent

Strangles is caused by infection with *Streptococcus equi* subspecies *equi* (*S. equi*), which, despite the inference from its name, is actually a sub-group of the diverse population of *Streptococcus equi* subspecies *zooepidemicus* (*S. zooepidemicus*) with which it shares over 97% DNA identity[3]. Non-equine infections with *S. zooepidemicus* can be severe, including cases of acute fatal hemorrhagic pneumonia in dogs[4-6] and septicemia, meningitis and toxic shock syndrome in humans[7-10]. However, despite its ability to cause severe clinical signs and its close relationship with *S. equi*, *S. zooepidemicus* is often regarded as a commensal organism of the equine upper respiratory tract. *S. zooepidemicus* is associated with a variety of diseases in horses including uterine infections of mares[11,12] and ulcerative keratitis[13]. Investigations of outbreaks of respiratory disease due to *S. zooepidemicus* have been confounded by the diversity of this group of bacteria and the ability of outbreak strains to persistently infect the tonsils of recovered horses[14]. Emerging evidence supports epidemiological studies that suggested a causal role for *S. zooepidemicus* in cases of respiratory disease[15] and modern typing methods[16] have directly linked specific strains to individual outbreaks[17,18]. Such data indicates that the population of *S. zooepidemicus*, which is resident in the tonsils of horses, includes pathogenic strains and provides a snapshot of the history of infection within an individual animal[19]. The differentiation of *S. equi* from the resident population of *S. zooepidemicus* and the immune responses to these closely related pathogens is a particular challenge for modern diagnostic techniques and is described in more detail below.

Clinical signs of strangles

Strangles is characterized by pyrexia, followed by abscessation of lymph nodes in the head and neck[20]. The name strangles was coined from the signs of dysphagia that some horses experience. Indeed, some of these horses are literally suffocated by the enlarged lymph nodes, which can obstruct the airway. Affected animals develop pharyngitis, which may lead

to them being reluctant to eat, particularly dried food, resulting in anorexia. Some affected horses stand with their neck extended and depression is common.

Following entry of *S. equi* via the nose or mouth, the organism attaches to and invades the tonsillar crypts of the oro- and nasopharynx and can be detected in the lymph nodes of the head and neck within three hours post-infection[21]. The transient attachment of *S. equi* to the oro- and nasopharynx before invasion into adjacent lymphoid tissue is highlighted by an inability to detect *S. equi* using nasopharyngeal swabs or washes taken 24 hours post-infection. Clusters of *S. equi* are apparent in the *lamina propria* after 48 hours[21]. Superantigens[22,23], phospholipase A_2 toxins[3], streptolysin S[24] and several other surface and secreted proteins[25-29] produced by *S. equi* modulate the proliferation and activity of these neutrophils leading to a failure of innate immune defences[21]. For example, the SeM surface protein is known to bind fibrinogen and immunoglobulin, providing an increased resistance to phagocytosis[25,30-34].

Lymph node abscesses increase in size and develop a thick fibrous capsule that walls off the infection. Growth of the bacterium in these abscesses may be increased through the production of a secreted molecule, equibactin, which enhances the capacity of *S. equi* to import iron[35]. Bacterial proliferation and an ineffective immune response induce a rise in body temperature. The onset of pyrexia can vary from a few days to several weeks post-infection depending on the infectious dose received and one individual animal to another, but can usually be detected before bacteria begin to shed from infected lymph nodes. Therefore, once a strangles outbreak is confirmed, presumptively infected pyretic horses may be identified and isolated before the organism is passed to in-contact animals. Normally fever persists and increases, exceeding 42^{o}C (107.6°F) in some cases, as abscesses mature in infected lymph nodes.

Abscesses formed in the retropharyngeal or submandibular lymph nodes typically rupture between 7 days and four weeks post-infection. Retropharyngeal lymph node abscesses

usually rupture into the guttural pouches, which drain via the eustachian tube into the nasopharynx resulting in the profuse mucopurulent nasal discharge typically associated with cases of strangles. Abscesses may also form in the cervical and tracheal/bronchial lymph nodes, rupturing externally through the skin over a process of several weeks. *S. equi* is particularly resistant to phagocytosis and killing by the equine immune system[25,26,28,36,37] and the process of abscess rupture that permits drainage of purulent material is important for the resolution of the infection. Older horses often exhibit a milder atypical form of the disease, possibly as a result of cross/partial-protection due to prior infection with different strains of *S. zooepidemicus* or *S. equi*[38] or through differences in the infecting strain[39,40].

Convalescence and the carrier state

Despite the severity of clinical signs during the acute phase of disease, the vast majority of horses (~98%) recover from strangles over a period of weeks. An adaptive immune response can be detected two-weeks post-infection[41], assisting mucosal clearance of *S. equi*[42]. An estimated 75% of recovered horses develop protective immunity to *S. equi*[43,44]. However, despite the development of antibody responses, approximately 10% of convalescent horses fail to clear all abscess material from their guttural pouches or sinus tract. Residual pus dries and hardens to form chondroids that can remain in the horse for several years and potentially the remaining lifetime of that animal[45,46]. Live *S. equi* persists in chondroids, or possibly as a biofilm on mucosal surfaces, and can intermittently shed from 'carrier' animals into the environment.

S. equi does not survive for long in the environment, particularly on surfaces exposed to direct sunlight[47]. However, *S. equi* shed from an acutely or persistently infected individual may gain access to naïve horses via the nose or mouth, through contaminated drinking water (in which it can persist for up to one month), tack, and other fomites. The ability of *S. equi* to establish persistent infection is critical to inter-epizootic transmission, the recurrence of strangles, and the high incidence of this disease around the world.

Therapeutic treatment of horses with strangles

Although *S. equi* is sensitive to all antibiotics, with the exception of aminoglycosides, veterinary opinion remains divided as to whether antibiotic treatment is useful. On identification of an index case, the isolation of healthy in-contact animals and the immediate administration of antibiotics to them for 3 to 5 days may prevent these animals from developing clinical signs of disease. However, treated animals are unlikely to develop immunity to *S. equi* infection and remain susceptible[38], and the use of antibiotics in horses with subclinical infection where abscesses have already formed only delays the onset of clinical signs and extends the time taken to resolve the outbreak.

The use of antibiotic therapy may provide temporary clinical improvement in fever and lethargy, which may assist the management of severe cases, particularly if the animal presents with dyspnea as a result of partial upper airway obstruction. Penicillin is considered the drug of choice and antibiotic resistance has not yet been reported in *S. equi*. However, antibiotic resistance has begun to emerge in some strains of *S. zooepidemicus*[5] and appropriate consideration should be given before sanctioning their use.

Therapeutic treatment of persistently infected carriers

Elimination of *S. equi* from the guttural pouches of persistently infected horses can be accomplished by endoscopic guttural pouch lavage. Sedation aids in implementation of endoscopy and facilitates drainage of flush material from the guttural pouches by lowering the horse's head. Chondroids present in the guttural pouch can be removed using a memory-helical polyp retrieval basket through the biopsy channel of the endoscope (Box 1)[46]. Surgical hyovertebrotomy and ventral drainage through Viborg's triangle carries inherent risks of general anesthesia and surgical dissection around major blood vessels and nerves and *S. equi* contamination of the hospital environment, but is practical in the event that large numbers of chondroids are identified within the guttural pouch. Empyema of the

guttural pouch can be resolved by repeated lavages with isotonic saline or polyionic fluid using rigid or indwelling catheters or through the use of a suction pump attached to the endoscope. Topical installation of 20% (w/v) acetylcysteine solution may assist the treatment of empyema. Topical benzylpenicillin (Box 1) is instilled into the guttural pouches via an endoscope guided into the pouch opening. The administration of systemic antibiotics may further improve treatment success. The guttural pouches are re-sampled two weeks later to confirm lack of infection following analysis by qPCR.

Complications

Bastard strangles occurs when the infection spreads to lymph nodes or tissues distant from the lymph nodes of the head and neck, and can be difficult to diagnose. A history of exposure to *S. equi* and laboratory results consistent with chronic infection, anemia, fever responsive to penicillin, hyperfibrinogenemia, and hyperglobulinemia, are supportive of the diagnosis of metastatic abscessation. Treatment requires long-term antimicrobial therapy, and appropriate local treatment or drainage of abscesses if possible. However, metastatic infection often results in the death of the affected animal, particularly when abscesses form in the lungs, liver, spleen, kidneys or brain.

Purpura hemorrhagica is an aseptic necrotizing vasculitis resulting in edema of the head, ventral abdomen and limbs, and petechial hemorrhages of the mucous membranes. Although often associated with *S. equi* infection, purpura hemorrhagica is believed to be caused by the deposition of immune complexes in blood vessels and can occur in response to several different antigens[48], including an excessive anti-SeM antibody response[49]. Treatment usually consists of dexamethasone and supportive care, including the administration of intravenous fluids, hydrotherapy, and bandaging. Mortality rates of between 8% and 25% have been documented, but most horses can recover from purpura given good veterinary care[48-50].

196 **Preventing infection**

197 Recent research has catalogued the genetic differences between *S. equi* and *S.*
198 *zooepidemicus* strains[3]. Although much of this work lies outside the current review,
199 knowledge of the *S. equi* genome is shedding new light on how this organism causes
200 disease[51,52] and has enabled the identification of novel targets leading to the development of
201 fast, sensitive and specific diagnostic tests[41,53,54] and new preventative vaccines[55].
202
203 **Culture test for *S. equi***

204 The diagnosis of *S. equi* infection has traditionally relied upon the inoculation of blood agar
205 containing colistin and nalidixic acid with clinical material recovered from swabs, washes or
206 abscesses and overnight incubation at 37^{o}C in a 5% CO_2 atmosphere. β-haemolytic colonies
207 of *S. equi* are picked and used to inoculate Todd Hewitt nutrient broth, which is incubated
208 overnight at 37^{o}C in a 5% CO_2 atmosphere. Finally, the turbid cultures are used to inoculate
209 purple broth cultures containing trehalose, lactose or sorbitol. *S. equi* fails to ferment these
210 sugars, whilst *S. zooepidemicus* usually ferments lactose and sorbitol and *Streptococcus*
211 *dysgalactiae* subspecies *equisimilis* (*S. equisimilis*), another common β-haemolytic
212 streptococcus, ferments trehalose[56]. Consequently, the isolation and identification of *S. equi*
213 using this method is time consuming and requires a minimum of 48 hours from receipt of
214 clinical samples. This reporting delay often has consequences for the isolation of infected
215 horses, providing *S. equi* with greater opportunity to transmit through naïve populations. The
216 isolation of *S. equi* is confounded by the presence of other β-haemolytic bacteria, most
217 notably *S. zooepidemicus* and *S. equisimilis*. Advances in polymerase chain reaction (PCR)
218 technology have highlighted deficiencies in the culture test, demonstrating that it is no longer
219 the gold standard method for the detection of *S. equi* or diagnosis of strangles.
220
221 **PCR assays**

222 The first PCR-based tests developed for *S. equi* targeted the 5' region of the SeM gene and
223 were estimated to be around three-times more sensitive than the traditional culture

assay[57,58]. However, this region is highly variable[39,40] and some strains of *S. equi* isolated from persistently infected carriers lack the target region[40,59]. Advances in PCR technology have led to the development of quantitative PCR (qPCR) assays. These assays can be completed in less than 2 hours from sample receipt and benefit from even greater levels of sensitivity. The superantigen-encoding genes have been exploited as diagnostic targets for the detection of *S. equi* by qPCR[53]. However, there is a certain level of functional redundancy in *S. equi*[3,23] that permitted the loss of at least one of these target genes in an outbreak of strangles identified in the USA (R. Holland personal communication).

Recently, a triplex qPCR assay has been developed[54] that targets two *S. equi*-specific genes, *eqbE*, encoding part of the equibactin biosynthesis system[3,35] and SEQ2190 encoding a unique surface protein[3]. An internal control strain of *S. zooepidemicus* is added to the clinical sample prior to DNA extraction to serve as a within-assay control to eliminate the risk of false-negative reporting through failures in DNA extraction or the presence of PCR inhibitors. The triplex assay has an overall sensitivity of 93.9% and specificity of 96.6% and detects ten-fold fewer quantities of *S. equi* than the limit of the culture assay, regardless of the presence of contaminating bacteria[54].

The culture assay failed to identify 39.7% of qPCR positive samples. In the past, the poor sensitivity of the culture assay and its failure to correctly identify qPCR positive samples was excused by the claim that PCR detects “dead DNA”[38]. Whilst technically this is correct and qPCR can detect killed *S. equi* following insufficient cleaning and sterilization of endoscopy equipment, DNA does not persist on mucosal surfaces *in vivo* and any culture or qPCR positive result should be taken seriously (Box 2). The presence of contaminating β-haemolytic streptococci explained 56% of triplex-positive/culture-negative results, and poor assay sensitivity explained the remaining 44% of false negative culture results reported by Webb *et al.* 2013[54]. Therefore, the triplex qPCR sets a new benchmark for quality control

and sensitivity and is now regarded as the new gold-standard test for the detection of *S. equi*.

Serology tests for exposure to *S. equi*

Persistence of *S. equi* in the guttural pouches of horses is associated with follicular hyperplasia[60], suggesting that it may be possible to identify persistently infected carriers in the absence of bacterial shedding through the quantification of a specific antibody response.

Vaccination with SeM-containing vaccines has been linked with complications such as purpura hemorrhagica[48] and this perceived risk 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[38]. The SeM iELISA has subsequently been exploited for the identification of horses infected with *S. equi*, with high antibody titers being suggested to indicate disseminated disease[38]. However, the SeM protein has a homologue in *S. zooepidemicus*, SzM[40], which raises the possibility that antibodies directed against SzM could cross-react with the SeM iELISA leading to the identification of false-positive horses. Pre-incubating sera with heat-killed *S. zooepidemicus* removes cross-reactive antibodies to SzM prior to the detection of SeM-specific antibody responses[61]. However, this process, although successful in reducing assay background, has not been adopted in assays based on full length SeM.

To overcome the problem of cross-reactivity with *S. zooepidemicus*, an iELISA assay using the N-terminal portion of SeM, which is unique to *S. equi*, has been developed[41]. The new assay is performed alongside a second iELISA to quantify the levels of antibodies against the *S. equi*-specific portion of SEQ2190 and a positive result is issued if either or both of the iELISAs exceed the positive cut-off[41]. Comparison of the dual antigen iELISA with a commercial iELISA marketed by IDvet (which is based on the full SeM protein) demonstrated that although the IDvet iELISA had comparable sensitivity (89.9% vs. 93.3%), it incorrectly

identified 23% of negative sera as being positive when they originated from the Icelandic horse biobank at Keldur, which had no possibility of containing *S. equi*-specific antibodies. In contrast, the dual iELISA yielded a specificity of 99.3%, highlighting its application to identify potentially infected animals before they can transmit the infection[41].

A prototype of the dual iELISA test, based on antigen A (SEQ2190) and antigen B, has previously been used to determine the prevalence of exposure to *S. equi* in different horse populations. A study of 109 horses in Lesotho identified eleven seropositive horses (10.1%)[62]. Another study identified 133 seropositive animals amongst 319 healthy horses (42%) at 31 unregulated events and yards in Ireland[63]. Some yards had no seropositive horses, while the prevalence at others was as high as 90%, highlighting premises with endemic infection (Box 3). The iELISA also identified all ten horses that had recently suffered from clinical signs of strangles in a UK study[64].

Utilizing the diagnostic tests to minimize the impact of *S. equi* infection

Persistently infected horses appear clinically normal and may remain unaffected during outbreaks of disease. Following resolution of clinical signs in affected animals, the screening of unaffected animals is often regarded as an unnecessary expense. However, if left untreated these animals remain a source of future infection.

The lack of clinical signs exhibited by persistently infected carriers emphasizes the need to implement effective quarantine and testing procedures for their identification and treatment before they come into contact with an existing herd. The quarantine area should be separated from the rest of the premises and clearly marked equipment (brushes, water buckets, etc.) should be used to maintain biosecurity. Regular disinfection of equipment and water can minimize the opportunity for *S. equi* to persist in the environment. Horses in quarantine should ideally be attended only by dedicated staff that do not deal with other horses, or failing that, be seen only by staff after they have dealt with other horses in order to

minimize transmission of any infectious agent from potentially infectious quarantined animals. Body temperatures should be obtained twice daily to identify signs of pyrexia at the earliest opportunity, which can then be investigated further. A blood sample taken on arrival can be used to identify recently exposed or persistently infected horses (Box 4). If negative, a second blood sample taken two weeks later should be tested to identify horses that have seroconverted and may have been incubating the infection. If this is also negative and the horse remains free from clinical disease, then it should be safe to enter the herd. Horses testing positive via the blood test should be investigated further. Ideally, the guttural pouches should be visually examined by endoscopy to identify obvious signs of persistent infection and a saline wash should be taken for analysis by qPCR. If qPCR tests on these samples are negative then it should be safe for the horse to enter the herd. If any of the samples test qPCR positive or chondroids are visible on endoscopy, then these should be treated as described above before entry onto the premises.

The first clinical signs of strangles: pyrexia, nasal discharge and enlarged submandibular lymph nodes can be highly variable in appearance from one horse to another and are not restricted to *S. equi* infection. However, if *S. equi* infection is suspected, the horse should be isolated immediately to minimize the risk of transmission to in contact animals. A needle aspirate from an enlarged or abscessed lymph node is the optimal sample for confirmation of *S. equi* infection. *S. equi* rapidly invades the lymph nodes of infected horses and is often not isolated from nasal swabs or washes taken during the early stages of disease[38], so a negative nasal swab/wash result by culture or even qPCR does not necessarily mean that the animal is not infected with *S. equi* – particularly if clinical signs suggest otherwise. Create three color-coded groups, even if limited space dictates that horses must remain in the same paddock only separated by two layers of electric fence to avoid nose to nose contact. The red group includes horses that have shown one or more clinical signs consistent with strangles. Amber group horses are those that have had direct or indirect contact with an infected horse in the red group and may be incubating the infection. The remaining green

group horses have had no known direct or indirect contact with affected animals. The body temperature of all horses in the green and amber groups should be obtained twice daily and any febrile horse should be moved to the red group. Color code buckets and other equipment to ensure that mixing between groups does not occur and wherever possible use dedicated staff for each color-coded group. If separate staff are not an option, staff should always move from the lowest risk to highest risk groups i.e. green to amber to red groups in that order and not back again. No horses should be allowed in, or out, of the yard at this time.

Screening procedures to identify those horses persistently infected with *S. equi* should commence no sooner than three weeks post resolution of the last clinical case. Screening of horses in the amber and green groups using the dual iELISA test will identify other horses that were exposed before or during the outbreak that could be sub-clinical carriers, which if left untreated could trigger subsequent outbreaks (Box 5). Animals testing positive by iELISA and those in the red group should be investigated by guttural pouch endoscopy, or if this is not possible, nasopharyngeal swabbing/washing to establish if they are persistently infected with *S. equi*. Samples should be tested by qPCR to maximize sensitivity and carriers treated as described above.

Vaccination against strangles

The horse is one of the most widely travelled animals on the planet and vaccination could play an important role in protecting horses from the inadvertent exposure to *S. equi* whilst attending equine events or sales. Therefore, the ideal strangles vaccine would confer adequate levels of protection against the currently circulating strains of *S. equi* with a long duration of immunity. Most equine vaccinations are administered via intramuscular injection and so, ideally, a strangles vaccine should be safe to administer via this route. It should be possible to differentiate infected from vaccinated animals (DIVA capability) in order for the vaccine to be used alongside existing management strategies which incorporate qPCR and

iELISA diagnostic tests. DIVA not only enables the normal movement of vaccinated horses, but also permits the identification of vaccinated horses that were exposed to and successfully protected from *S. equi*. Such data would build confidence in a vaccine, facilitating its wider use leading to increased herd immunity.

Killed and cell extract strangles vaccines

The first documented vaccine against strangles was developed by Bazeley working with the Australian military in the 1940s[65-69]. His vaccine was based on a culture of *S. equi*, which was heat-killed at 55^{o}C for 12 minutes and administered subcutaneously. Severe injection site reactions and pyrexia were frequently observed in vaccinated animals. However, 29 of approximately 2,500 vaccinated horses developed strangles compared with 101 of approximately 1,900 unvaccinated animals (P <0.0001)[67]. Analysis of formalin-killed, or *S. equi* extracts failed to demonstrate protection in this study[67].

Cell-free versions of this early vaccine are available in some parts of the world and include Equivac S (Zoetis New Zealand), Strepguard (MSD Animal Health) and Strepvax II (Boehringer Ingelheim), which are administered by the intramuscular route. However, very little data on the efficacy of these vaccines is publically available. One study found that 17 of 59 (29%) foals vaccinated with an SeM-based vaccine and 39 of 55 (71%) controls had clinical signs of strangles when observed 2 weeks post third vaccination (P <0.0001). However, 32 of 60 (53%) foals vaccinated with the same vaccine and 29 of 60 (48%) controls had clinical signs of strangles (P = 0.72) when observed 6 weeks post third vaccination. These data suggest that any protection conferred by this vaccine was short-lived. Furthermore, 44% and 29% of vaccinates, respectively, developed adverse reactions at the injection site in this study[70]. None of these vaccines have DIVA capability.

Live attenuated vaccines

390 The only strangles vaccine available in Europe is Equilis StrepE (MSD Animal Health).
391 Equilis StrepE is a live-attenuated *aroA* deletion mutant, which is based on a 1990 isolate
392 from Holland[40,71]. In two separate studies in which all non-vaccinated control animals
393 developed strangles, two doses of 10^9 colony forming units (cfu) of Equilis StrepE
394 administered via submucosal (SM) injection into the upper lip protected 5/5 and 2/4 horses
395 from developing lymph node abscesses following intra-nasal challenge two weeks post
396 second SM vaccination (P = 0.0476 and P = 0.4286, respectively)[71]. Intramuscular (IM)
397 administration of this vaccine appeared to be efficacious, protecting all three vaccinated
398 animals[71]. However, injection site reactions, from which the vaccine strain was recovered,
399 precluded administration via this more conventional and convenient route[40,71,72]. Adverse
400 reactions following SM vaccination with Equilis StrepE have been reported[72]. The vaccine
401 contains the same genetic material as virulent strains of *S. equi* (excluding *aroA*), and so
402 interferes with culture and qPCR tests whilst the vaccine strain persists, and iELISA tests by
403 triggering positive test results that cannot readily be differentiated from those arising from
404 natural infection. The lack of DIVA capability confounds the identification of vaccinated
405 horses that are infected with virulent strains of *S. equi*. Therefore, all vaccinated animals
406 triggering a positive iELISA result are required to be examined further to eliminate the
407 possibility that they may be persistently infected (Box 6).
408
409 A second live-attenuated vaccine, Pinnacle IN (Zoetis), for intranasal (IN) administration, is
410 available in the USA and some other territories. The vaccine is based on the CF32 strain
411 that was isolated from a horse in New York during 1981 and attenuated via treatment with
412 nitrosoguanidine. As with Equilis StrepE, Pinnacle causes adverse effects if injected
413 intramuscularly and does not have DIVA capability. The vaccine strain has been linked to
414 lymph node abscesses and can be shed up to 46 days post-vaccination of young (<1 year-
415 old) ponies[73]. Furthermore, *S. equi* resembling the Pinnacle IN vaccine strain was isolated
416 from recently vaccinated horses in New Zealand that had subsequently developed strangles,
417 suggesting that some horses may have increased sensitivity to the vaccine, or that the strain

418 can revert to virulence[74]. No data on efficacy has been published, but at the 'Getting to Grips
419 with Strangles' meeting in Stockholm, 2010, Zoetis stated that following experimental
420 challenge 9/15 controls developed strangles compared with 3/22 high dose (P = 0.0049) and
421 2/22 low dose (P = 0.0023) vaccinates 3 weeks post V2. Therefore, the commercial live-
422 attenuated vaccines for strangles can confer significant levels of protection. However, they
423 lack DIVA capability and have been linked to adverse reactions in some animals.
424
425 Early research data suggested that all *S. equi* strains were identical as sera from a
426 convalescent horse cross-reacted with other isolates and there was no variation in *Hind*III
427 restriction pattern between different *S. equi* isolates on Southern blot analysis using an SeM
428 gene probe[34]. However, sequence analysis of the SeM gene identified differences between
429 strains of *S. equi*[2,39,40,74,75], with 128 different alleles currently identifiable in the online
430 database http://pubmlst.org/cgi-bin/mlstdbnet/agdbnet.pl?file=sz_seM.xml (last accessed
431 10th April 2014). Evidence suggests that the population of *S. equi* is changing over time as
432 the organism continues to evolve, with domination of SeM-9 strains of *S. equi* within the
433 UK[2,75]. Equilis StrepE and Pinnacle were derived from strains that cluster into groups of *S.*
434 *equi* distantly related to the dominant SeM-9 strains. Although an antibody response is likely
435 to cross-react between different strains of *S. equi*[34], the level of protection conferred by
436 these vaccines against currently circulating strains of *S. equi* remains unknown.
437
438 **Subunit vaccines:**
439 Subunit vaccines are based on recombinant *S. equi* proteins produced in and purified from
440 *Escherichia coli* strains. These vaccines generally have an excellent safety profile as only
441 the desired target proteins are used in the vaccine. Subunit vaccines do not contain *S. equi*
442 DNA and can be designed to avoid the particular antigens used in diagnostic tests,
443 conferring the ability to differentiate infected from vaccinated animals (DIVA). However, the
444 identification of protective antigens represents a significant challenge for vaccine design.
445 The vaccination of mice with recombinant SeM conferred protection against challenge with

S. equi[76], but these promising results were not repeated following the vaccination and challenge of horses[77]. Two subunit vaccines consisting of six *S. equi*-specific proteins or five *S. equi* adhesin proteins also failed to confer protection in ponies, despite the generation of promising serum antibody responses[78]. However, a combination of seven *S. equi* surface and secreted proteins, Septavac, which were identified through analysis of the *S. equi* genome[3] and mouse studies[79], protected 6 of 7 vaccinated Welsh mountain ponies two weeks post-third vaccination (P = 0.0047)[55]. The inclusion of the immunoglobulin-cleaving proteins IdeE and IdeE2 in the vaccine were found to be important to efficacy and a five-component vaccine, Pentavac, lacking these components protected only one of seven ponies two weeks post forth vaccination[55]. The combination of proteins used did not include SeM or SEQ2190 and so this vaccine is likely to have DIVA capability[41]. The Septavac vaccine (now known as Strangvac) is based on a SeM-9 strain of *S. equi* recovered from a horse in Sweden in 2000[55], which is more closely related to the dominant strains circulating the UK horse population.

Summary and future perspectives

S. equi has evolved to exploit the anatomy of the horse producing abscesses in the lymph nodes, which enable the organism to persistently infect a proportion of convalescent animals. Shedding of *S. equi* from carrier animals enables the onward transmission of this pathogen and further outbreaks of disease. Therefore, the identification and treatment of persistently infected carriers is critical if the cycle of infection is to be broken and *S. equi* eradicated. The improvements to the available diagnostic tests greatly assist the identification of persistently infected animals and are preventing new outbreaks of disease. However, further work is required to permit their use alongside effective vaccines, which can increase herd immunity and reduce the number of strangles outbreaks occurring in horse populations around the World.

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Box 1: Preparation of a gelatin/penicillin solution for topical treatment of persistent infection of the guttural pouch

Weigh out 2 g of gelatin and add 40 mL sterile water.

Heat or microwave to dissolve the gelatin.

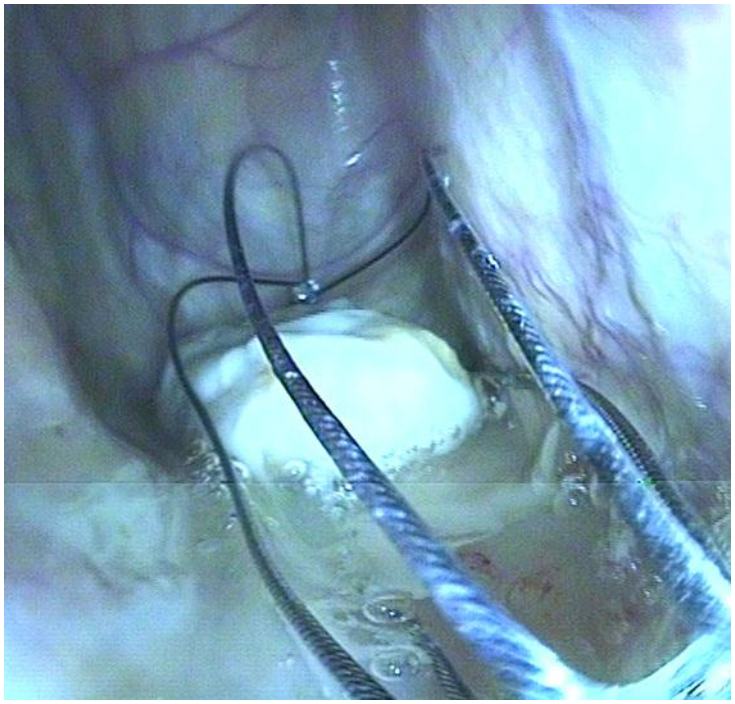
Cool gelatin to 45 to 50 ^{o}C.

Add 10 mL sterile water to 10,000,000 units (10 Mega units) sodium benzylpenicillin G.

Mix penicillin solution with the cooled gelatin to make a total volume of 50 mL.

Dispense into syringes and leave overnight at 4 ^{o}C to set.

Figure 1: Recovery of a chondroid from the guttural pouch of a horse using a memory-helical polyp retrieval basket through the biopsy channel of the endoscope.

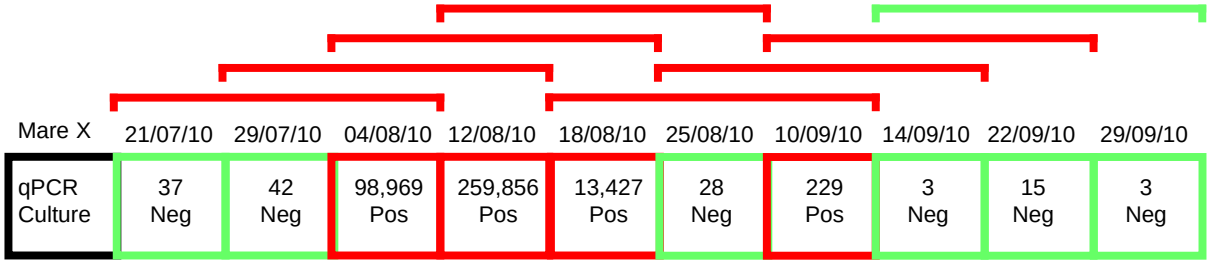


Box 2: Example case report:

A convalescent mare was sampled at weekly intervals by nasopharyngeal swab and the sample analyzed by both culture and qPCR. The third sample taken on the 4th August 2010

tested positive by both qPCR and culture (Figure 2), but despite advice to the attending veterinarian to examine the mare by guttural pouch endoscopy, the horse was resampled at weekly intervals until the 29th September 2010 when three consecutive negative nasopharyngeal swab samples had finally been obtained. The horse was pronounced infection-free and permitted to move to a new yard where a new outbreak of strangles began on the 25th October 2010. In this example, the qPCR and culture assays agreed precisely and highlight the intermittent nature of shedding S. equi from the guttural pouch. This case also emphasizes the need to examine positive horses by guttural pouch endoscopy to ensure that persistently infected horses are not inadvertently missed.

Figure 2. Culture and qPCR results following sampling of a convalescent mare over time.

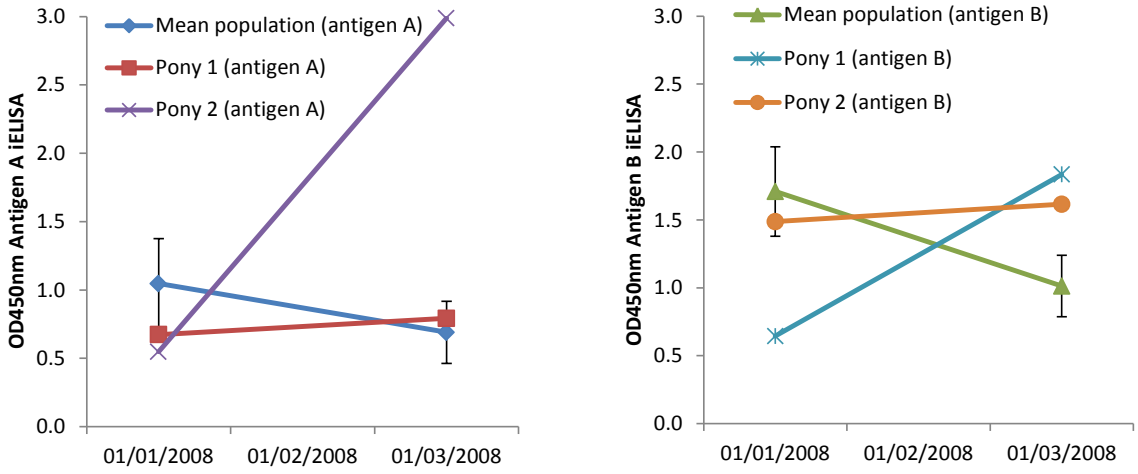


<u>*Box 3: Example case report:*</u>

A yard of 52 polo ponies had recurrent problems with strangles including cases in August 2007, 8th and 13th December 2007 and 5th and 16th January 2008. However, the clinical signs were generally mild. The AHT iELISA was used to screen 48 resident horses sampled on the 17th January 2008, identifying 39 (81%) as seropositive. Forty ponies from this population were sampled again eight weeks later, showing a general decline in antibody levels to both antigen A and antigen B (Figure 3), although 20 (50%) remained seropositive. However, antibody levels in two ponies (pony 1 and pony 2) that had suffered from pyrexia and mucopurulent nasal discharge from the 5th and 16th January 2008, respectively increased,

providing evidence that the iELISA was successfully detecting genuine exposure to and infection with S. equi. Further investigation of six seropositive healthy ponies with no history of clinical signs of strangles by guttural pouch endoscopy identified a persistently infected carrier, highlighting the endemic status of S. equi infection on this yard.

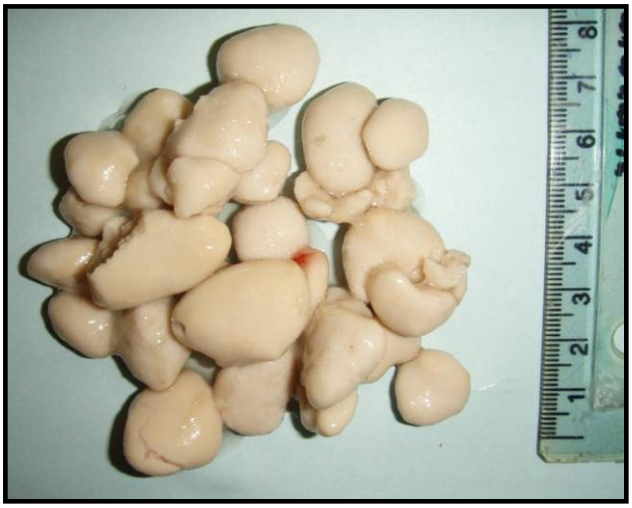
Figure 3: Seroconversion of two clinically affected polo ponies whilst resident on a yard with endemic S. equi infection. Error bars indicate the 95% confidence interval. A positive antigen A result is ≥ 0.5 OD_{450nm} and a positive result for the antigen B iELISA is ≥ 1.0 OD_{450nm}.



<u>*Box 4: Example case report:*</u>

Seven of 56 newly acquired horses arriving at a rescue center were found to be seropositive and later confirmed to be persistently infected by guttural pouch endoscopy and lavage (Figure 4). All carriers were treated and no clinical cases of strangles occurred on mixing with resident horses.

Figure 4: Twenty eight chondroids recovered from a healthy Shetland pony with serology assay OD_{450nm} of 2.5 for antigen A and 3.9 for antigen C.



Box 5: Example case report:

An outbreak of strangles in livery yard in Scotland of unknown source resolved and all horses and ponies were screened using the dual iELISA to identify unaffected horses for further investigation, alongside those affected in the outbreak. Guttural pouch endoscopy confirmed that a healthy Shetland pony was persistently infected with S. equi. The pony was treated and the infection eradicated.

Box 6: Example case report:

A pregnant mare in good health tested positive using the dual iELISA described above, despite the owner having no knowledge of a prior history of strangles or exposure to S. equi. The horse had been vaccinated five years previously with Equilis StrepE. However, it was decided that it was unlikely that the observed antibody response was due to vaccination. The mare was examined further by guttural pouch endoscopy and lavage. Washes recovered from the guttural pouches tested qPCR positive, indicating persistent infection. On further discussion with the owner it became clear that the horse had been stationed at the Defence

762 *Animal Centre at Melton Mowbray, UK at the time of a strangles outbreak. The mare was*
763 *treated to eliminate the persistent infection in the guttural pouch, which was confirmed by*
764 *qPCR analysis of guttural pouch lavages two weeks later.*
765